

# The Development of Azadirachtin as a Soil-Applied, Granular Insecticide

by

Gordon Wilson Scarlett Daly B.Sc. (Hons.) D.I.C. M.Sc.

Thesis submitted for the Degree of Doctor of Philosophy



**UNIVERSITY**  
*of*  
**GLASGOW**

February 2004

Division of Biochemistry and Molecular Biology

Institute of Biomedical and Life Sciences

University of Glasgow

## **Acknowledgements**

I would like to express my deepest gratitude to my supervisor Dr Robin Strang, whose scientific support and much needed sense of humour have been invaluable throughout this project. Without his expertise, this research would not have been possible. I also wish to express my sincerest thanks towards my industrial supervisor Dr Mark Cole, whose friendly encouragement, technical ability and foresight ensured that all areas of this project ran smoothly. Additionally, I am indebted to Dr Hugh Flowers whose expertise in soil science was essential during azadirachtin environmental fate studies. Without his aid, many of azadirachtin's physical/chemical characteristics could not have been elucidated.

I would like to thank Dr David McCracken for ensuring the facilities at the Scottish Agricultural College (Ayr) were available for the research purposes of this project. Similarly, I must also thank Mr Brian Laird for ensuring that these facilities remained in excellent working order.

Finally, I wish to take this opportunity to thank my wife Nicole, whose love, encouragement and support has kept me going throughout the last three years.

This project was funded by the Biotechnology and Biological Sciences Research Council (Polaris House, North Star Avenue, Swindon, SN2 1UH) and NeemCo Ltd. (2 Brewster Place, Irvine, Ayrshire, KA11 5DD).

## **Declaration**

I hereby declare that the thesis which follows is my own composition, that it is a record of work done by myself and that it has not been presented in any previous application for a Higher Degree.

A handwritten signature in black ink, consisting of a large, stylized 'S' followed by a series of loops and a long horizontal stroke extending to the right.

Gordon W. S. Daly

## **Abstract**

Azadirachtin, isolated from neem seed kernels, has increasingly been used as a botanically derived insecticide, which produces multiple toxic effects in insects. As a commercial product, azadirachtin is typically formulated as liquid sprays for foliar application. However, on leaf surfaces azadirachtin exhibits poor insecticidal contact activity and a rapid degradation by photolysis. Therefore, opportunities exist for the introduction of alternative strategies, which may improve the environmental and biological efficiency of applications. In this respect, the aim of this project was to develop azadirachtin as an insecticide that is applied to the soil, using a granular formulation, for root uptake and subsequent systemic plant protection.

A method was developed whereby azadirachtin could be rapidly isolated to approximately 95% purity using flash chromatography. This material was used in all subsequent chemical and biochemical studies. To increase the speed of crude extract analysis, a colorimetric technique was assessed to rapidly quantify azadirachtin. However, this method was generally unsuitable for the requirements of this project because it was non-specific and not stable.

Granular formulations based on sodium alginate, starch-kaolin and poly( $\epsilon$ -caprolactone), and containing different neem seed extracts were successfully prepared. These granules exhibited differences in the rate of azadirachtin release into water. Additives such as kaolin clay and rapeseed oil could be used to modify the speed of release. Following application to soil, the position of granules did not affect release rates. However, granule application method was shown to affect the rate at which the limonoid was accumulated within the nasturtium plants.

Azadirachtin was shown to be moderately water-soluble (1.29 g/l). During mixing studies between distilled water and *n*-octanol, the limonoid partitioned more favourably into the non-aqueous phase at a ratio of 7:1. Based on calculated  $K_{oc}$  values ( $<40$ ), azadirachtin was classified as very highly soil mobile. Adsorption occurred principally to the organic matter of soils. Clay minerals were comparably non-sorbent. Desorption from both of these sites occurred readily.

Azadirachtin was not persistent within soil where the limonoid's  $DT_{50}$  was as short as 1.06 days. Initial breakdown resulted in the acetyl moiety being cleaved from the molecule. In addition, azadirachtin was shown to exhibit a pH sensitive hydrolytic degradation. The limonoid's half-life in solution ranged from 57 days at pH 5 to 7.15 hours at pH 9.

Azadirachtin was compared to the radiolabelled derivative [ $^3H$ ]-dihydroazadirachtin. Both molecules were shown to be 96% similar in terms of water: lipid solubilities and the rates that nasturtium roots absorbed them. Therefore, [ $^3H$ ]-dihydroazadirachtin represented a suitable tracer to study azadirachtin's behaviour in soils and plants.

Within nasturtium plants, azadirachtin was both phloem-mobile and xylem-mobile and therefore, is ambimobile. Following root absorption, azadirachtin appeared to be uniformly accumulated throughout the leaf lamina. Within these tissues, azadirachtin was stable, with a half-life of approximately 9 weeks.

In conclusion, a suitable granule for a controlled-release of azadirachtin was developed. Additionally, the limonoid was shown to be sufficiently mobile within soils and plants for movement from a granule source to the leaf tissue. However, an extremely rapid breakdown suggests that azadirachtin might not be appropriate for application to the soil as a commercial insecticide.



## **Contents**

Acknowledgements	I
Declaration	II
Abstract	III
Contents	IV
List of Figures	XIII
<b>Chapter 1 – Introduction</b>	<b>1</b>
1.1. Pesticides in agriculture	2
1.1.1. Organochlorines	3
1.1.2. Organophosphates	4
1.1.3. Carbamates	5
1.1.4. Synthetic Pyrethroids	6
1.2. Problems associated with pesticide use	10
1.2.1. Pesticides and public health	10
1.2.2. Ecological and environmental risk of pesticides	12
1.2.3. Resistance to pesticides	13
1.2.4. The regulation of pesticides and plant protection products	15
1.3. Alternative technologies for plant protection	16
1.4. Botanical insecticides	17
1.5. The neem tree	18
1.6. Insecticidal components of neem	24
1.7. The limonoid azadirachtin	25
1.8. Azadirachtin chemistry	26

1.9.	Azadirachtin related limonoids	27
1.10.	Biological activity of azadirachtin and its related terpenoids on insects	31
1.10.1.	Antifeedancy	32
1.10.2.	Effect on metamorphosis/growth regulation & biological fitness costs	34
1.10.3.	Fecundity, egg-sterility and reproductive effects	36
1.10.4.	Orientation repellence	38
1.10.5.	Oviposition repellence	39
1.11.	Problems associated with neem	40
1.11.1.	Delayed effects of insecticidal action	41
1.11.2.	Variable and limited field performance	41
1.11.3.	Resistance	43
1.11.4.	Commercial production	45
1.11.5.	Off-target effects	47
1.11.6.	Safety towards mammals	48
1.12.	The formulation of neem as an insecticide	49
1.13.	Project aims	54

## **Chapter 2 – Azadirachtin Isolation and Quantitative Estimation of Neem**

<b>Seed Extracts</b>	<b>55</b>
2.1.	Azadirachtin isolation
2.1.1.	Introduction
2.1.2.	Aims
2.1.3.	Materials and methods

2.1.3.1.	General materials	60
2.1.4.	Preliminary procedure for azadirachtin-A isolation	60
2.1.4.1.	Preparation of semi-purified azadirachtin for chromatography	60
2.1.4.2.	Flash column chromatography	60
2.1.4.3.	Analytical high performance liquid chromatography using the Dionex HPLC system	61
2.1.4.4.	Thin layer chromatography	61
2.1.5.	Refined procedure for azadirachtin-A isolation	62
2.1.5.1.	Preparation of semi-purified azadirachtin for flash chromatography	62
2.1.5.2.	Flash column chromatography	62
2.1.5.3.	Partitioning with dichloromethane	62
2.1.5.4.	Analytical high performance liquid chromatography using the Dionex HPLC system	63
2.1.6.	Results	66
2.1.7.	Discussion	74
2.2.	Quantitative estimation of neem seed extracts	75
2.2.1.	Introduction	75
2.2.2.	Aims	76
2.2.3.	Materials	76
2.2.3.1.	General materials	76
2.2.4.	Experimental	77
2.2.4.1.	Extraction of azadirachtin and related limonoids from crude neem seed extracts	77

2.2.4.2.	Preparation of methanol extracts for the vanillin assay	77
2.2.4.3.	Preliminary vanillin assay	78
2.2.4.4.	Calibration curve of azadirachtin subjected to vanillin assay	78
2.2.4.5.	Finalised vanillin assay	79
2.2.4.6.	The vanillin assay using methanol	79
2.2.4.7.	Calculation of extinction coefficients	79
2.2.4.8.	Analytical high performance liquid chromatography using the Beckman HPLC system	80
2.2.5.	Results	81
2.2.5.1.	Analysis of pure limonoids and crude neem seed extracts by high performance liquid chromatography	81
2.2.5.2.	Analysis of pure limonoids and crude neem seed extracts by vanillin assay	82
2.2.6.	Discussion	104

## **Chapter 3 – The Development of a Controlled-Release Granule** 107

3.1.	Introduction	108
3.1.1.	Pesticide formulations	108
3.1.2.	Granular formulations	109
3.1.3.	Alginate granules	110
3.1.4.	Starch granules	111

3.1.5.	Poly( $\epsilon$ -caprolactone) granules	111
3.1.6.	Controlled-release of pesticides from granules	112
3.2.	Aims	116
3.3.	Materials and methods	116
3.3.1.	General Materials	116
3.4.	Experimental	117
3.4.1.	Production of sodium alginate granules	117
3.4.2.	Production of starch-kaolin granules	117
3.4.3.	Poly( $\epsilon$ -caprolactone) granules	118
3.4.4.	Measurement of granule size and weight	118
3.4.5.	Determination of azadirachtin and related limonoids within granules	119
3.4.6.	The release of azadirachtin and related limonoids from granular formulations	119
3.4.7.	Swelling study of granules	120
3.4.8.	The breakdown of azadirachtin and related limonoids within granular formulations after 28 days immersed in water	121
3.4.9.	The shelf life of sodium alginate granular formulations	121
3.4.10.	Analytical high performance liquid chromatography using the Beckman HPLC system	122
3.5.	Results	123
3.5.1.	Granule production	123
3.5.2.	The release of azadirachtin and related limonoids from granular formulations	139
3.5.3.	Granule swelling study	145



3.5.4.	The breakdown of azadirachtin and related limonoids within granular formulations after 28 days immersed in water	145
3.5.5.	The shelf life of sodium alginate granular formulations	146
3.6.	Discussion	155
3.6.1.	Granule production	155
3.6.2.	Granule release	156
3.6.3.	Formulation stability	161
<b>Chapter 4 – The Movement of Azadirachtin within Soil and Plant Systems</b>		<b>162</b>
4.1.	Introduction	163
4.1.1.	The movement of pesticides within soil	163
4.1.2.	Sorption and desorption of pesticides to soil	164
4.1.3.	Soil sorption coefficients	165
4.1.4.	The movement of pesticides within plants	166
4.1.5.	Movement within the vascular systems of plants	168
4.1.6.	The application of azadirachtin as a soil-applied, systemic insecticide	171
4.2.	Aims	172
4.3.	Materials	172
4.4.	Experimental	173
4.4.1.	Radiolabelling of azadirachtin	173
4.4.2.	Preliminary azadirachtin water solubility study	173
4.4.3.	Final azadirachtin water solubility study	173
4.4.4.	Aqueous hydrolysis of [ <sup>3</sup> H]-dihydroazadirachtin	174

4.4.5.	N-octanol/water partition coefficient of azadirachtin and [ <sup>3</sup> H]-dihydroazadirachtin	175
4.4.6.	Determination of compost organic matter and organic carbon content	176
4.4.7.	Determination of soil moisture content at a soil water potential of -0.5 bar	176
4.4.8.	Adsorption of [ <sup>3</sup> H]-dihydroazadirachtin to soils and peat	177
4.4.9.	Desorption of [ <sup>3</sup> H]-dihydroazadirachtin from soils and peat	177
4.4.10.	Breakdown of azadirachtin within soil	178
4.4.11.	Plant growth conditions	179
4.4.12.	Root uptake and translocation of [ <sup>3</sup> H]-dihydroazadirachtin from granules applied to compost	179
4.4.13.	Collection of [ <sup>3</sup> H]-dihydroazadirachtin from xylem vessels	180
4.4.14.	Collection of [ <sup>3</sup> H]-dihydroazadirachtin from phloem vessels	180
4.4.15.	Distribution of [ <sup>3</sup> H]-dihydroazadirachtin within the leaf lamina	181
4.4.16.	Metabolism of [ <sup>3</sup> H]-dihydroazadirachtin within plants	181
4.4.17.	Specific activity of [ <sup>3</sup> H]-dihydroazadirachtin	182
4.4.18.	Loading of Nasturtium plants with [ <sup>3</sup> H]-dihydroazadirachtin	182
4.4.19.	Extraction of azadirachtin and [ <sup>3</sup> H]-dihydroazadirachtin from water	183
4.4.20.	Extraction of [ <sup>3</sup> H]-dihydroazadirachtin from soil or plant material	183
4.4.21.	Authoradiography	183
4.4.22.	Scintillation counting	184

4.4.23.	Analytical high performance liquid chromatography using the Beckman HPLC system	184
4.4.24.	Preparative high performance liquid chromatography	185
4.4.25.	Thin layer chromatography (system 1)	185
4.4.26.	Thin layer chromatography (system 2)	185
4.5.	Results	186
4.5.1.	Solubility of azadirachtin and [ <sup>3</sup> H]-dihydroazadirachtin	186
4.5.2.	Aqueous hydrolysis of [ <sup>3</sup> H]-dihydroazadirachtin	193
4.5.3	Adsorption and desorption of [ <sup>3</sup> H]-dihydroazadirachtin with soils and peat	193
4.5.4.	Breakdown of azadirachtin within soil	201
4.5.5.	Root uptake and translocation of [ <sup>3</sup> H]-dihydroazadirachtin from soil applied granules	202
4.5.6.	The behaviour of [ <sup>3</sup> H]-dihydroazadirachtin within plants	214
4.6.	Discussion	221
4.6.1.	A comparison of azadirachtin with [ <sup>3</sup> H]-dihydroazadirachtin	221
4.6.2.	Aqueous solubility of azadirachtin and [ <sup>3</sup> H]-dihydroazadirachtin	223
4.6.3.	Adsorption of azadirachtin to organic matter and clay minerals	223
4.6.4.	Desorption of azadirachtin from organic matter and clay minerals	227
4.6.5.	Sorption coefficients of azadirachtin	228
4.6.6.	Persistence of azadirachtin within soils	230
4.6.7.	Identification of the azadirachtin metabolites in soil	233

4.6.8.	Aqueous Hydrolysis of [ $^3\text{H}$ ]-dihydroazadirachtin	235
4.6.9.	Root uptake and systemic distribution of [ $^3\text{H}$ ]- dihydroazadirachtin from soil applied granules (part 1)	237
4.6.10.	Root uptake and systemic distribution of [ $^3\text{H}$ ]- dihydroazadirachtin from soil applied granules (part 2)	239
4.6.11.	The xylem and phloem mobility of azadirachtin	241
4.6.12.	The distribution of [ $^3\text{H}$ ]-dihydroazadirachtin within the leaf lamina	243
4.6.13.	The metabolism of [ $^3\text{H}$ ]-dihydroazadirachtin within plants	244
<b>Chapter 5 – Final Discussion and Conclusions</b>		247
5.1.	Final discussion	248
5.1.1.	Project overview	248
5.2.	Chapter 2 overview – Azadirachtin isolation and quantitative estimation of neem seed extracts	248
5.2.1.	Isolation of azadirachtin	248
5.2.2.	Quantitative estimation of neem seed extracts	249
5.3.	Chapter 3 overview – The development of a controlled-release granule	251
5.4.	Chapter 4 overview – The movement of azadirachtin within soil and plant systems	252
5.4.1.	The behaviour of azadirachtin within soils	252
5.4.2.	The behaviour of azadirachtin within plants	253
5.4.3.	The movement of azadirachtin from granule to leaf tissues	255
5.5.	Concluding remarks	255
5.6	Conclusion	260
<b>References</b>		261

## **List of Figures**

### **Chapter 1 – Introduction**

Figure 1.1.	Structural examples from the four major insecticide classes.	8
Figure 1.5.1.	Photographs of the neem tree (1).	20
Figure 1.5.2.	Photographs of the neem tree (2).	22
Figure 1.9.	The major triterpenoids of neem and their parent steroidal intermediate, tirucallol.	29
Figure 1.12.	Examples of neem/azadirachtin based commercial formulations.	52

### **Chapter 2 – Azadirachtin Isolation and Quantitative Estimation of Neem**

#### **Extracts**

Figure 2.1.1.	Preparative clean-up phase of azadirachtin-A isolation.	58
Figure 2.1.5.	Refined procedure for azadirachtin-A isolation.	64
Figure 2.1.6.1.	Analysis of azadirachtin-A and related limonoids eluting from flash column.	68
Figure 2.1.6.2.	Purity of azadirachtin-A eluting from flash column.	70
Figure 2.1.6.3.	Purified azadirachtin-A resulting from flash column chromatography.	72
Figure 2.2.5.1.	HPLC chromatograms of the major limonoids of neem.	86
Figure 2.2.5.2.	HPLC chromatograms of crude neem seed extracts.	88
Figure 2.2.5.3.	Absorbance spectra of major neem limonoids subjected to vanillin assay.	90



Figure 2.2.5.4.	The response of the major neem limonoids to the vanillin assay.	92
Figure 2.2.5.5.	Absorbance spectra of crude neem seed extracts (1).	94
Figure 2.2.5.6.	Absorbance spectra of crude neem seed extracts (2).	96
Figure 2.2.5.7.	Azadirachtin-A calibration curve.	98
Figure 2.2.5.8.	Concentration of azadirachtin and related limonoids within neem seed extracts, as estimated by the vanillin assay.	100
Figure 2.2.5.9.	Concentration of azadirachtin, nimbin and salannin within neem seed extracts, as estimated by HPLC.	102

### **Chapter 3 – The Development of a Controlled-Release Granule**

Figure 3.1.	Categorisation of polymeric systems used for controlled-release.	114
Figure 3.5.1.	Photographs of sodium alginate granules (1).	125
Figure 3.5.2.	Photographs of sodium alginate granules (2).	127
Figure 3.5.3.	Photographs of starch-kaolin granules.	129
Figure 3.5.4.	Photographs of poly( $\epsilon$ -caprolactone) granules.	131
Figure 3.5.5.	Hydrated granule sizes and weights.	133
Figure 3.5.6.	Dried granule sizes and weights.	135
Figure 3.5.7.	Concentration of azadirachtin and related limonoids within granules.	137
Figure 3.5.8.	The release of azadirachtin and related limonoids from granular formulations.	141

Figure 3.5.9.	Azadirachtin and related limonoids within, and released from, granular formulations.	143
Figure 3.5.10.	Granule (weight) swelling study.	147
Figure 3.5.11.	Granule (diameter) swelling study.	149
Figure 3.5.12.	The breakdown of azadirachtin and related limonoids within granular formulations after 28 days immersed in water.	151
Figure 3.5.13.	The shelf life of sodium alginate granular formulations.	153
 <b>Chapter 4 – The Movement of Azadirachtin within Soil and Plant Systems</b>		
Figure 4.5.1.	Soil characteristics.	187
Figure 4.5.2.	Azadirachtin water solubility.	189
Figure 4.5.3.	Comparison of azadirachtin with [ <sup>3</sup> H]-dihydroazadirachtin.	191
Figure 4.5.4.	Aqueous hydrolysis of [ <sup>3</sup> H]-dihydroazadirachtin.	195
Figure 4.5.5.	Adsorption and desorption of [ <sup>3</sup> H]-dihydroazadirachtin with soils and peat.	197
Figure 4.5.6.	Soil sorption and soil organic carbon sorption coefficients for [ <sup>3</sup> H]-dihydroazadirachtin.	199
Figure 4.5.7.	Breakdown of azadirachtin within soils (1).	204
Figure 4.5.8.	Breakdown of azadirachtin within soils (2).	206
Figure 4.5.9.	Breakdown of azadirachtin within soils (3).	208
Figure 4.5.10.	Isolation of the main azadirachtin breakdown product.	210
Figure 4.5.11.	Root uptake and translocation of [ <sup>3</sup> H]-dihydroazadirachtin from soil applied granules.	212

Figure 4.5.12.	Transport of [ $^3\text{H}$ ]-dihydroazadirachtin within the vascular systems of plants.	215
Figure 4.5.13.	The distribution of [ $^3\text{H}$ ]-dihydroazadirachtin within the leaf lamina.	217
Figure 4.5.14.	Metabolism of [ $^3\text{H}$ ]-dihydroazadirachtin within plants.	219

# Chapter 1

## Introduction

## **1.1. Pesticides in agriculture**

Approximately one third of the world's food crop is damaged or destroyed by insect pests during growth, harvest and storage (Jacobson, 1988). The term "pest" originates from the Latin word *pestis*, meaning plague or contagious disease, and in modern terminology has been defined by Hill (1994) as any insect or organism causing harm to humans or livestock, or damage to crops, cultivated plants or possessions.

In agriculture there are several important consequences of insect attack. Firstly, damage (to crop plants, or harvested parts), followed by a loss in yield (both of quality and quantity), and eventually a monetary loss. Fenemore (1984) classified insect related damage into two distinct categories – direct and indirect. On a crop basis, direct damage is when solid portions of tissue are removed from harvestable plant parts, resulting in holes and tunnels in leaves, stems and fruits. In contrast, indirect damage describes the consequences of infection through insect transmission of plant viruses, fungi, bacteria and toxic or irritant saliva. These may result in the stunting, distortion or death in areas (necrosis) of plant tissue (Stapley & Gayner, 1969).

Approximately 10,000 species of insect are regarded as pests of agricultural crops and of these around 600 produce damage that requires the use of control measures (Schwartz & Klassen, 1981). Historically, these measures were limited to the modification of physical and cultural practices, such as crop rotations, trap crops and adjustments of planting dates. Nevertheless, severe yield losses were still common following heavy pest infestations (Bernard *et al.*, 1997).



As a result, to improve crop productivity, poisonous chemicals were employed. Prior to the 1930s, these materials consisted of simple preparations of lime, copper or sulphur (Robinson & Sutherland, 2002). However, following the discovery of the insecticidal properties of DDT (dichlorodiphenyltrichloroethane) in 1939, crop protection was revolutionised (Vieirra *et al.*, 2001). DDT represented the first member of the organochlorine class of insecticides. Following the compound's widespread adoption and success, a number of related structures were synthesised, such as the benzene hexachloride group, as well as the insecticides dieldrin, chlordane, heptachlor and mirex (Smith & Gangolli, 2002).

Later, advances in chemical techniques brought about the development of new insecticidal classes and today more than one thousand active ingredients are produced and used world-wide (Aprea *et al.*, 2002 and Leake, 2000). These materials have found applications in agriculture, in commercial and industrial activities, domestically and in government applications and are dominated by four major classes of compounds (Abou-Arab, 2002). These are the organochlorines, organophosphates, carbamates and synthetic pyrethroids.

### 1.1.1. Organochlorines

Organochlorines are a broad class of pesticides that can be divided into 3 groups. These are, the benzene hexachloride isomers (e.g. lindane), cyclodienes (e.g. aldrin, chlordane, endosulfan) and DDT (figure 1.1.A.) and its analogues (methoxychlor, dicofol, chlorobenzylate) (Aprea *et al.*, 2002).

These compounds were the first major class of synthetic organic chemicals to become widely used as insecticides (Plapp, 1981). However, as a consequence of persistent environmental problems and health hazards, their manufacture and use was subsequently discontinued in many developed countries during the 1970s. Nevertheless, they still find uses in various developing countries for public health purposes (e.g. Malaria eradication in Vietnam) (Kunisue *et al.*, 2003).

The precise mode of action of organochlorine insecticides remains unclear. Nevertheless, studies have indicated that they generally act as nervous system stimulants. However, the nature of this effect does appear to be influenced by the specific active substance. For example, DDT appears to interfere with axonic transmission of nerve impulses, whereas cyclodienes act by interfering with acetylcholine release from presynaptic vesicles. In addition, other members of the group have been shown to impair the function of the nervous system by altering the balance between excitatory and inhibitory thresholds in neurons (Colosio *et al.*, 2003 and Plapp, 1981). Despite these differences, poisoning from this group causes insects to undergo multiple uncontrolled muscular spasms resulting in permanent muscle contraction (Hassall, 1969).

### 1.1.2. Organophosphates

Organophosphate pesticides are a diverse group of highly toxic chemicals, most of which are esters and thioesters of phosphoric and thiophosphoric acid (Liu *et al.*, 2001 and Storm *et al.*, 2000). Since their discovery in the 1940s, approximately 500,000 organophosphates have been evaluated for commercial use (Sales *et al.*, 2000)

Members of this group (e.g. chlorpyrifos (figure 1.1.B.), dichlorvos and malathion) have been used extensively in agriculture and in certain residential applications, where in many situations they have replaced organochlorine pesticides (Kousba *et al.*, 2003 and Liu *et al.*, 2001).

The mode of action of organophosphates is well documented. Most disrupt normal synaptic function in the insect nervous system by inhibiting the enzyme acetylcholinesterase. Inhibition by these compounds occurs either directly, or more commonly, after their accumulation (Byrne & Toscano, 2002 and Zhu & Gao, 1999). Through this, the hydrolysis of the neurotransmitter acetylcholine is prevented which leads to subsequent hyper-polarisation of the post-synaptic membrane (Galloway *et al.*, 2002 and Hamers *et al.*, 2003). Ultimately, poisoning by organophosphates leads to lethal dysfunction in treated insects (Villatte & Bachmann, 2001).

### 1.1.3. Carbamates

Carbamates are synthetic derivatives of physostigmine (eserine), which is a pharmacologically active principle isolated from the calabar bean, *Physostigma venenosum* (Coats, 1994). These compounds are highly effective insecticides used in numerous soil or plant treatments (Sánchez-Brunete *et al.*, 2003). Members of the group (e.g. carbofuran (figure 1.1.C.), carbaryl and propoxur) are extensively applied in urban, agricultural and aquacultural situations and along with organophosphates represent approximately 50% of the total insecticide and acaricide usage worldwide (Galloway *et al.*, 2002 and Villatte & Bachmann, 2001).

Carbamates bear a spatial, structural resemblance to acetylcholine (Hassall, 1969). Consequently, their insecticidal mode of action is well understood and they have been shown to act in a similar manner to organophosphates, inhibiting acetylcholinesterase resulting in nervous system disruption (Sanchez-Hernandez & Walker, 2000 and Yerushalmi & Cohen, 2002). However, unlike organophosphate compounds, inhibition in this instance is of short duration and is reversible (Aprea *et al.*, 2002).

#### 1.1.4. Synthetic Pyrethroids

The synthetic pyrethroids are regarded as one of the most important classes of insecticide, contributing to over 25% of the world insecticide market (Vais *et al.*, 2001). These compounds have found numerous uses in agriculture, silviculture and in urban situations, and in combination with the organophosphates, have largely replaced the organochlorine insecticide class (Best & Ruthren, 1995 and Fakata *et al.*, 1998).

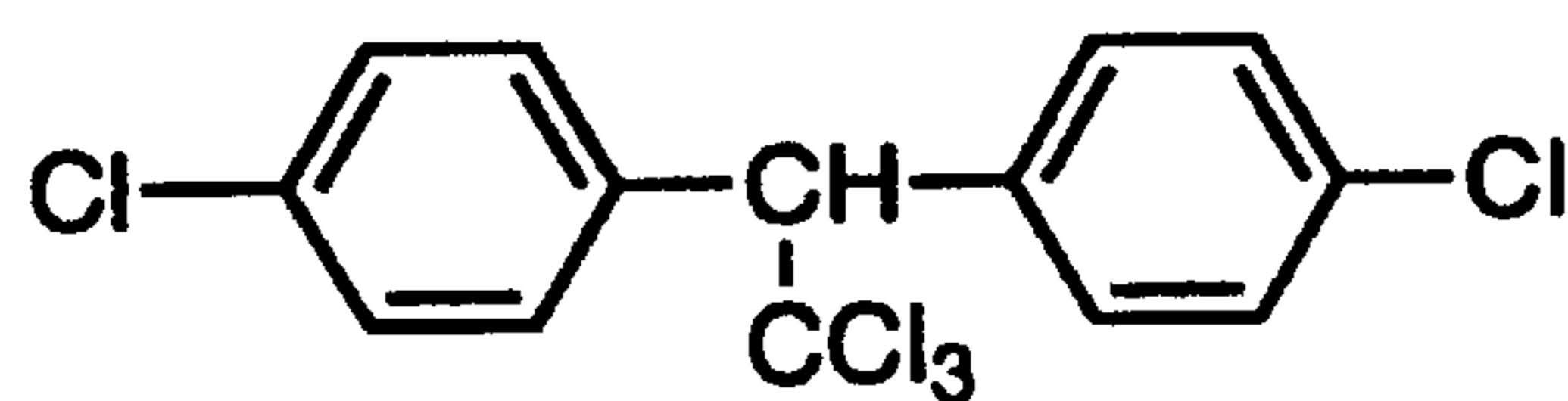
These materials are photostable, synthetic derivatives of the natural insecticide pyrethrum and exhibit improved environmental persistence through the addition of single or multiple halogen atoms within the molecular structure (Coats, 1990 and Plapp, 1981). The class may be divided into two groups. These are the type I pyrethroids (e.g. permethrin), which lack a cyano moiety in the  $\alpha$ -benzylic position, and type II pyrethroids (e.g. deltamethrin (figure 1.1.D.)), which have this  $\alpha$ -cyano group (Narahashi, 2000).

Pyrethroids tend to be fast-acting, and like DDT act on the insect nervous system through sodium channel modulation. Specifically, sodium currents are prolonged,

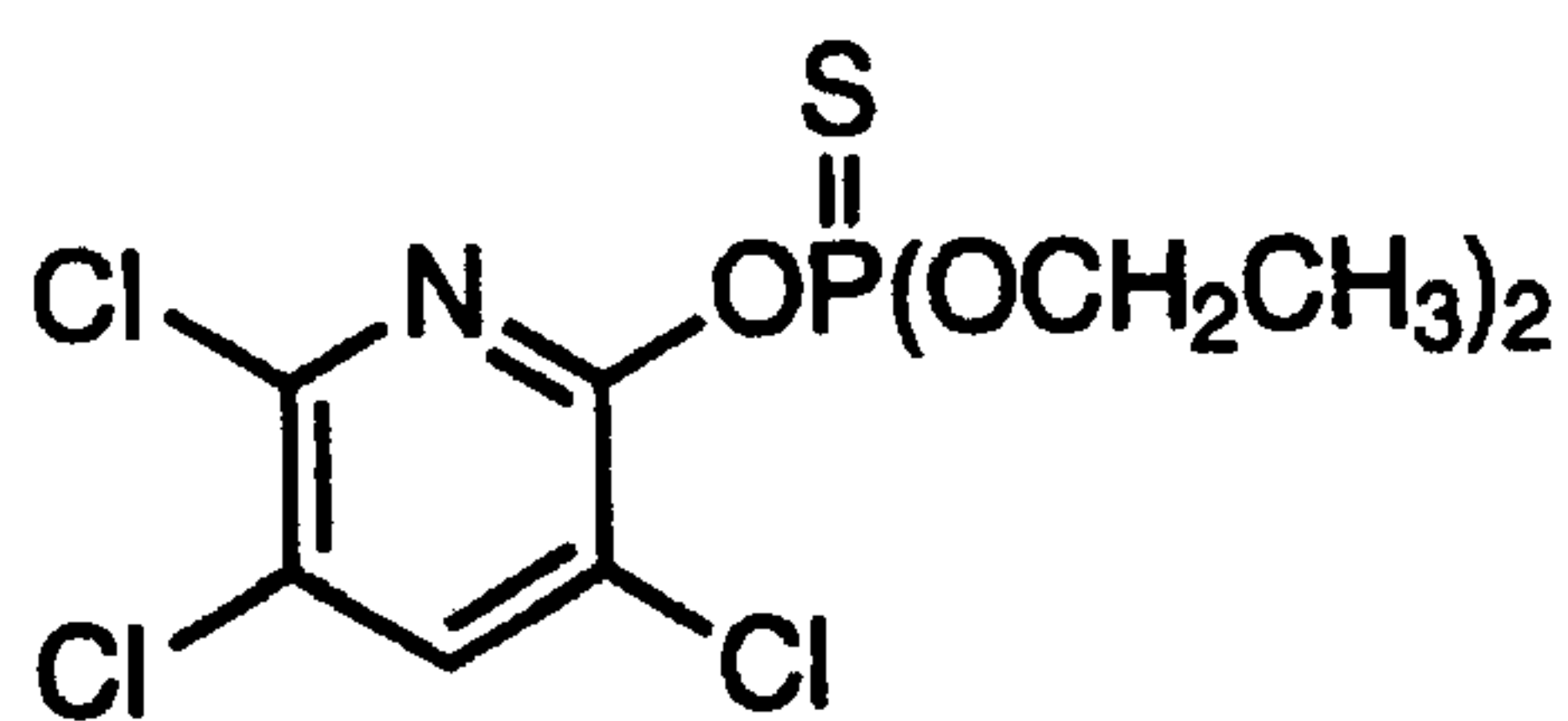
resulting in the depolarising after-potential of a nerve impulse being extended and elevated, which produces repetitive after-discharges. Ultimately, this effect causes hyperexcitation (i.e. increased activity and restlessness), ataxia (i.e. loss of coordination and prostration), convulsions, tremors and eventual paralysis in poisoned insects (Narahashi, 2000; Plapp, 1981; Toth & Sparks, 1990 and Vais *et al.*, 2001).



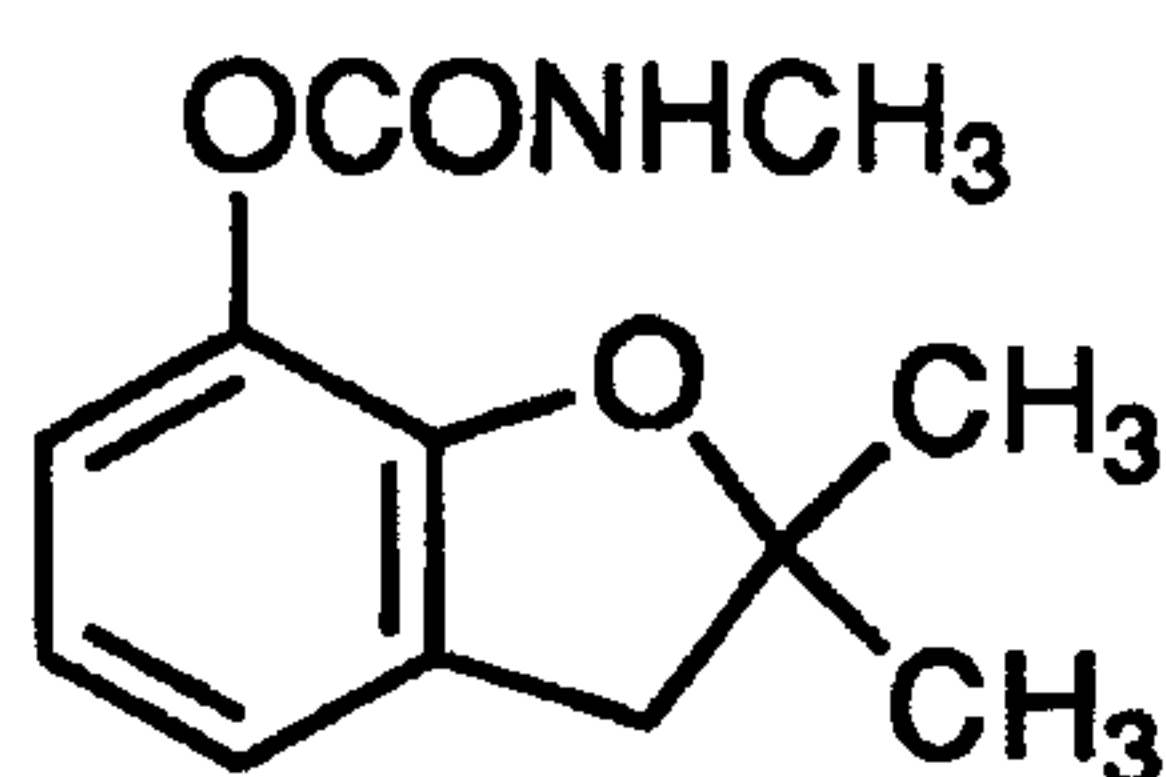
Figure 1.1. Structural examples from the four major insecticide classes (Tomlin, 1994).



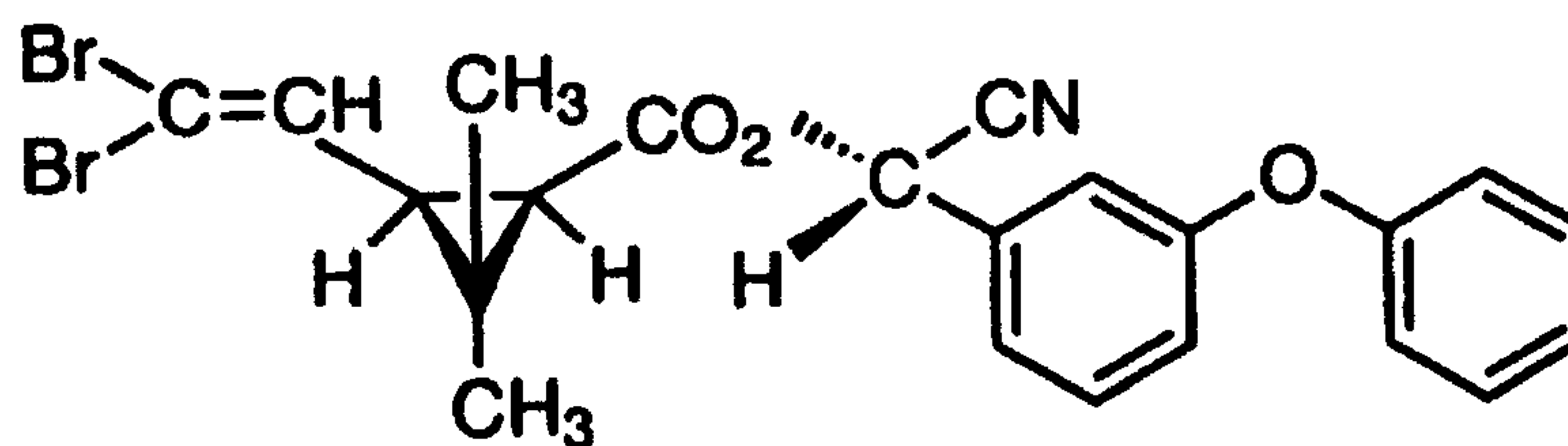
(A) DDT (Organochlorine)



(B) Chlorpyrifos (Organophosphate)



(C) Carbofuran (Carbamate)



(D) Deltamethrin (Synthetic pyrethroid)

## **1.2. Problems associated with pesticide use**

Pesticides have played a crucial role in increasing agricultural productivity. Without technologies such as these, Avery (1997) estimated that tripling world crop yields between 1960 and 1992 (in line with increasing demands of the human population) would have required the cultivation of an additional 25 – 30 million square kilometres of land. However, despite their success, pesticide use has come with associated costs.

Pesticides are unique among toxic chemical substances as they are deliberately released into the environment (Lewalter & Leng, 1999). However, since their toxicity may not be completely specific for the target organism, their use may pose a public health as well as an ecological and environmental risk (Colosio *et al.*, 2003 and Pimentel *et al.*, 1981). In addition, the development of resistance, due to repeated applications, has caused reductions or even complete losses of efficacy (Brattsten, 1989). These concerns are now being addressed in current legislative issues (e.g. Council Directive 91/414/EEC, 1991), which are shaping today's agrochemical industry.

### **1.2.1. Pesticides and public health**

Humans are exposed to poisons from a number of sources, such as tobacco, alcohol, waste gases and from various pharmaceutical agents. However, the long-term health implications associated with exposure to pesticides and their residues are often perceived as a greater hazard (Coats, 1994 and Garcia *et al.*, 2000). Nevertheless,

risks related to unintentional pesticide exposure are often overstated, with poisonings being rare and generally short-lived events (Cochran, 2002 and Keifer, 2000).

The implications of pesticide exposure on human health have yet to be comprehensively documented and may involve a number of different exposure scenarios (Banerjee, 1999 and Renwick, 2002). During urban and agricultural use of pesticides, exposure typically occurs through various manual activities (e.g. mixing and application) with skin being the primary route of uptake (Ross *et al.*, 2000). However, ingestion (through poor work practices) and inhalation (through application of highly volatile materials) are two less common yet significant routes of occupational exposure (Banks *et al.*, 1990).

Because pesticide residues may remain on agricultural commodities, dietary intake of material is thought to be the predominant source of non-occupational pesticide exposure (Heudorf & Angerer, 2001 and Krol *et al.*, 2000). However, pesticide residues are frequently recorded being transported into the home, therefore dermal routes can contribute to significant uptake, particularly in young children following contact with substances on floors and other surfaces (Nishioka *et al.*, 1996).

Following exposure, pesticides may acutely or chronically influence human health. Acute poisoning occurs rapidly after the exposure of some bodily process to direct chemical action. However, more commonly poisonous effects are of a chronic nature occurring after long-term, low-level exposures (Banks *et al.*, 1990; Bernard *et al.*, 1997 and Ritter, 1997).

The symptoms of toxicity, particularly chronic types, can be diverse and vary according to the specific pesticide and the level of exposure (van der Werf, 1996 and Vidal *et al.*, 2002). Nevertheless, signs of toxicity include neurotoxic disorders (i.e. damage to the nervous system, for example hyperexcitability, convulsions and paralysis), immunodisfunction (i.e. reduction or alteration in immune responses, for example neoplasm, allergy and autoimmunity), mutagenesis (i.e. genetic changes, for example chromosomal aberrations, sister-chromatid exchanges and micronuclei), teratogenesis (i.e. production of malformations or deviations from the normal type, for example birth defects in the male reproductive tract) and carcinogenesis (i.e. cancer promotion, for example prostate, bladder and breast cancer as well as non-Hodgkin's lymphoma) (Ballantyne, 1992; Banerjee, 1999; Bernard *et al.*, 1997; Bolognesi & Morasso, 2000; Colosio *et al.*, 1999; Gómez-Arroyo *et al.*, 2000; Laden *et al.*, 2001; Longnecker, 1997; Lotti, 2002; Nishioka *et al.*, 1999; Settini *et al.*, 2003; Vale *et al.*, 2003; van der Werf, 1996 and Webster *et al.*, 2002).

### 1.2.2. Ecological and environmental risk of pesticides

Pesticides pose a risk to not only human health but also towards the health of the environment due to their long persistence and high potential for bioaccumulation. Therefore, they have been observed influencing a wide range of natural processes, yet the extent of their impact is often unclear (Archer & Shogren, 2001; Kakareka, 2002 and Wilson & Tisdell, 2001). Nevertheless, the environmental consequence of pesticide use depends on the degree of exposure (i.e. dispersion and resulting environmental concentration) and on the toxicological properties of the chemical concerned (van der Werf, 1996).



Each year approximately 2.5 million tons of pesticides are applied to agricultural crops and in some cases less than 0.3% of the applied pesticide reaches the target pest (van der Werf, 1996). Inadvertent translocation and deposition often occurs via spray drift, soil re-suspension and volatilisation. These translocation mechanisms combined with phase redistribution, long range transport and desorption from air to water, soil, fog, rain and snow, have been implicated in wide-scale contamination of the ecosystem (Hernández *et al.*, 2000; Nishioka *et al.*, 1996 and Unsworth *et al.*, 1999).

Following their application, pesticides can damage soil structure and nutrient status (through toxic action on soil fauna and flora), impact on bird populations (through reproductive failure or suppression and physical malformations), damage aquatic life (through direct toxicity, elimination of essential foods or by reducing dissolved oxygen levels), diminish crop productivity (through toxicity towards beneficial insects such as honeybees and natural enemies of insect pests) and poison terrestrial wildlife (through consumption of contaminated prey or by direct contact with residues) (Ayas *et al.*, 1997; Barnett *et al.*, 2002; Kleivane *et al.*, 2000; Konstantinou, 2000; Pimentel *et al.*, 1992; Stickel *et al.*, 1984; Stoate *et al.*, 2001; van der Werf, 1996 and Wilson & Tisdell, 2001).

### 1.2.3. Resistance to pesticides

Resistance is regarded as one of the most serious problems facing pesticide use and is defined as a failure to control pests under field conditions with formerly effective doses. This can lead to increased application rates, increased pesticide use and

ultimately a loss of efficacy, resulting in increased risk to users, consumers and the environment (Heimbach *et al.*, 2002; Rotteveel *et al.*, 1997 and Scott *et al.*, 2000).

Resistance falls into two categories. These are cross-resistance (resistance to chemically related pesticides through a common detoxification pathway) and multiple resistance (resistance to a variety of pesticide classes through the co-occurrence of several resistance mechanisms) (Metcalf, 1989). Regardless of category, resistance development is dependant on several factors, such as selection pressures (i.e. amount of pesticide used), levels of immigration of susceptible individuals (i.e. gene flow), relative dominance of resistance alleles and population structure (Roush & Daly, 1990). The mechanisms conferring resistance have been summarised by Lee & Lees (2001) and include behavioural modifications (i.e. avoidance of residues), biochemical alterations (i.e. changes in target site binding activity and enhanced pesticide detoxification), and physiological adaptations (i.e. alteration of absorption, excretion, transport or sequestration of substances).

Despite the problems associated with resistance, there is still no general agreement on what constitutes a strategy to combat its occurrence, although tackling the problem through prevention rather than cure seems the most suitable option (Clarke *et al.*, 1997). This philosophy has been adopted into integrated pest management programs which aim at long term sustainability through a combination of control regimes, thereby optimising the efficiency and profitability of crop production (Council Directive 91/414/EEC, 1991; Denyer, 2000 and Metcalf, 1989).

#### 1.2.4. The regulation of pesticides and plant protection products

Increasing awareness regarding the potential impacts of pesticide use has led to the development of legislative control in order to protect human health and the environment and to ensure that products are used correctly and efficiently (ECPA, 1998b and Neale, 2000). The primary regulatory instrument within the European Union is Council Directive 91/414/EEC (adopted in July 1991), which sets out a community-harmonised framework for authorisation, use and control of these products (ECPA, 2000a; ECPA, 2000b; SANCO, 2001).

The basic principle of the directive is the development of a positive list (Annex 1) of active substances through a review program. Fundamental to the review is that it is more important to protect human health, wildlife and the environment than to improve agricultural productivity (ECPA, 1998a and SANCO, 2001). The goal of this program has been to conduct an evaluation of over 800 existing active substances during a 12-year period (originally due for completion in July 2003, although now extended until at least 2008) while simultaneously assessing new active substances (ECPA, 2001a).

Ultimately, this has required manufacturers to “defend” their plant protection products at a considerable cost (€ 3.7 million for existing and € 33.6 million for new active substances). As a result, it is expected that this in combination with the increased regulatory demands will lead to approximately 490 substances (roughly 60% of the total available) being withdrawn by 2008 (Europa, 2002; Europa 2003 and ECPA, 2001b). Consequently, it has been predicted that there may be a deficit between the plant protection products needed and that which is available (SANCO, 2001).

### **1.3. Alternative technologies for plant protection**

Synthetic pesticides are often regarded as the only available tools, proven to significantly reduce pest populations to acceptable levels and as a consequence they have become heavily relied upon (Edelson *et al.*, 2002 and Margni *et al.*, 2002). However, as previously discussed, regulatory pressures, resistance and increased awareness regarding ecological, environmental and human health impacts have reduced their availability and promoted the search for safer alternatives (Warrior, 2000).

Consequently, in some cases, many of the crop protection products first used (e.g. lime, pyrethrum and nicotine) are being revisited as leads for new materials or for possible improvements by chemical modification. Simultaneously, newer technologies (e.g. engineering plants to express insecticidal proteins from *Bacillus thuringiensis*) are also being investigated (Betz *et al.*, 2000; Peterson *et al.*, 2000 and Thompson *et al.*, 2000).

Nevertheless, despite consumer resistance in some instances, current changes have led to increased opportunities for alternative control strategies and reduced risk pesticides. These include organic farming (which avoids synthetic fertiliser and pesticide use and promotes agricultural sustainability), transgenic crops (genetic manipulation to express transgenes that confer desirable traits), microbial organisms (which cause infection and disease, including viral, bacterial and fungal agents), entomopathogenic nematodes (which infest and kill through release of nematode symbiotic bacteria, including parasitic species from *Steinernema* and *Heterorhabditis* genera), insect



pheromones and semiochemicals (involving “lure and kill” and mating disruption methods), insect natural enemies (conservation biological control to increase predator and parasite populations), marine natural products (potential as possible lead compounds, include extracts from algae, sponges, corals and annelids) and botanical pesticides (plant essential oils and secondary metabolites that exhibit considerable insecticidal activity) (Copping & Enriz *et al.*, 2000; Menn, 2000; Dev & Koul, 1997; Edwards-Jones & Howells, 2001; Isman, 2000; McLaren, 2000; Peng *et al.*, 2003; Phillips, 1997; Powell & Picket, 2003; Schjønning *et al.*, 2002 and Warrior, 2000).

#### **1.4. Botanical insecticides**

Of the various alternatives, botanical pesticides have increasingly been studied due to historical successes (pyrethrum and nicotine are previous examples), high consumer acceptance (due to improved environmental safety compared to conventional pesticides), broad compatibility (i.e. applications in conventional and organic farming as well as home and garden purposes), diversity of insecticidal activity (e.g. feeding inhibition, oviposition inhibition, growth disruption, repellence, paralysis and death), number of potential sources (over 2,400 plants, belonging to more than sixty families) and number of possible insecticidal structures (10,000 secondary metabolites defined, estimated that over 400,000 exist) (Casanova *et al.*, 2002; Coats, 1994; Dev & Koul, 1997; Govindachari, 1992; McLaren, 1986 and Schoonhoven, 1982).

However, despite the considerable number of botanical insecticides, only four have been commercially used in crop protection. These are nicotine from tobacco leaves, rotenone from derris tree roots, pyrethrum from chrysanthemum flowers and

azadirachtin from the neem tree (Sugavanam & Copping, 1998). Of these, azadirachtin and pyrethrum are considered to be the most important botanical insecticides, accounting for approximately 1% of the global insecticide market (Isman, 2000). In addition, azadirachtin has continued to generate significant academic interest as research attempts to optimise and better understand the insecticidal potential of the compound (Yamasaki *et al.*, 1986).

### **1.5. The neem tree**

The Neem Tree, *Azadirachta indica* A. Juss (Syn. *Melia azadirachta*, *Antelaea azadirachta*) also known as the Indian Lilac or Margosa tree, belongs to the Meliaceae (mahogany) family (Jotwani & Srivastava, 1983; Koul, 1990 and Mulla & Cribb, 1999). The term neem or *nimba* is Sanskrit in origin and translates into “the healer and illness reliever” (Norten, 2000 and van der Nat, 1991).

Today the precise origins of the neem tree are still in dispute. Some authors have suggested that neem may have originated in southern and south-eastern Asia (Schmutterer, 2002 and Tedeschi *et al.*, 2001). However, morphological observations made by Oo (1987) cast doubt on these suggestions. Based on this work, the alternative proposal is that the tree is endemic to Burma and more specifically to the Union of Myanmar (Caboni, 2002). However, despite these findings the exact origin of the tree remains an area of some debate.

At the start of the 21<sup>st</sup> Century, *A. indica* has become widely distributed throughout India, South-East Asia, Australia, Africa, Central and South America (Rawat, 1995).



There have been several reasons for the trees widespread migration. *A. indica* has been used as a source of fuel, firewood, timber, herbal medicines, natural contraceptives and natural pesticides. However, the shade the tree imparts has proved to be an important incentive to its distribution and this quality is exploited to this day. Perhaps the best example of this is during the last 6 years with the planting of 50,000 trees in Mecca (Saudi Arabia) to provide shade for 2 million Muslim pilgrims during *haj* (Ascher, 1997 and Strang, personal communication).

The ecology of *A. indica* has been described by numerous authors. Individual trees may have a life expectancy exceeding 200 years and have been described as evergreen or deciduous (depending on environmental conditions), fast growing plants (66% of the total growth occurring in the first 3 years). These trees can reach girths of 2.5m, heights of 25m, and produce large circular or oval crowns. These crowns consist of unpaired, pinnate leaves ranging from 20-40cm long which are themselves comprised of smaller (3-8cm), medium to dark green, asymmetric leaflets. Neem has often been observed as sporadically flowering between September and October, although typically the tree will flower from January to May and produce whitish pink flowers that are 4-5mm in size and are pollinated by various species of Bee. Neem begins to bear fruit in 3-5 years and becomes fully productive after 10 years. Typically fruit is produced once a year, with total quantities ranging from 11-50kg per tree. These fruits are best described as oval, olive-like drupes, which when ripe produce a sweet, yellow pulp and a brown seed kernel (Ascher, 1997; Bengé, 1988; Puri, 1999; Radwanski, 1977 and Schmutterer, 1990).

Figure 1.5.1. **Photographs of the neem tree (1).** (A) Neem tree growing in the US Virgin Islands (photo [www.gotostcroix.com](http://www.gotostcroix.com)). (B) Asymmetrical leaflets of neem (photo [www.ibiblio.org](http://www.ibiblio.org)).



(A)



(B)





Figures 1.5.2. **Photographs of the neem tree (2).** (A) Fruits growing on neem tree (photo [www.fh-weihenstephan.de](http://www.fh-weihenstephan.de)). (B) Dried neem seed kernels (photo [www.glfc.forestry.ca](http://www.glfc.forestry.ca)).



(A)



(B)





### **1.6. Insecticidal components of neem**

Neem is one of the richest sources of secondary metabolites in nature (Schaaf *et al.*, 2000) and has long been recognised for its insecticidal and insect-repellent properties. Chopra (1928) was the first to report on the neem tree in this respect. However, it is only within that last 3-4 decades that significant investigations have been carried out in an attempt to isolate and identify the specific components, which give neem its remarkable biological activity. Yet to date, scientific knowledge of these fascinating and structurally complex molecules remains fragmentary.

While much emphasis has been placed on extracts from seed kernels, the leaves and bark of the tree have also yielded a number of active components (Fagoonee, 1986 and Mulla & Su, 1999). All of the well-characterised compounds identified in these extracts belong to the class of triterpenoids, which are derived through a series of oxidation and rearrangement reactions from the steroidal intermediate tirucallol (figure 1.9.A.). More specifically, those involved in the chemical defence of the tree can be regarded as limonoids and are defined as triterpene derivatives from which four side-chain carbon atoms (C24-C27) have been cleaved and the remainder have been cyclized to a furan ring, hence the alternative name tetranortriterpenoids (Govindachari, 1992 and Jones *et al.*, 1988).

Champagne *et al.*, (1986) has comprehensively reviewed the limonoid group. To date over 300 limonoids have been isolated and approximately 100 of these have been found to occur within the neem tree (Thejavathi, 1995). However, only a relatively small number (approximately 20) are reported as being biologically active against



insects (National Research Council, 1992). Nevertheless, of the various naturally occurring compounds screened for anti-insect activities in recent years, it is these limonoids that have shown the greatest potential for use in insect control (Hassanali & Bentley, 1986).

### **1.7. The limonoid azadirachtin**

Of the constituents isolated from the neem tree, the limonoid azadirachtin is considered to be the most important from both a commercial and biological perspective (Butterworth *et al.*, 1972; Champagne *et al.*, 1992; Jones *et al.*, 1988 and Schmutterer, 1990).

First isolated by Butterworth & Morgan (1968), azadirachtin became the focus of a scientific race to elucidate its molecular structure. This however proved to be a considerable technical challenge (Taylor, 1987). Butterworth *et al.*, (1972) correctly observed that azadirachtin contained a large number of functional groups. Consequently, because of this and the molecule's sensitivity to acids and bases, conventional structure analysis by proton and carbon nuclear magnetic resonance (NMR) proved difficult (Bilton *et al.*, 1987; Taylor, 1987 and Turner *et al.*, 1987).

Zanno *et al.*, (1975) was the first to present a complete structural assignment of azadirachtin. Their interpretations were based mainly on partially relaxed Fourier transform  $^{13}\text{C}$  NMR spectroscopy and a hypothetical relationship of azadirachtin with the known terpenoids salannin and nimbin. However, doubt persisted over several assignments that were not consistent with all NMR data (Kraus *et al.*, 1987).

It was another 10 years before azadirachtin's structure and absolute stereochemistry was unambiguously established by Kraus *et al.*, (1985). This research group used one-dimensional nuclear Overhauser effects (nOe) difference spectroscopy in conjunction with  $^{13}\text{C}$  deuterium isotope shift experiments for identification purposes. Simultaneously, Ley and co-workers derived the structure through x-ray crystallographic analysis of detigloyl-22, 23-dihydroazadirachtin (Broughton *et al.*, 1986).

### **1.8. Azadirachtin chemistry**

Rembold *et al.*, (1983) was the first to show that azadirachtin was composed of a mixture of congeners. Initially, data from NMR and circular dichroism (CD) showed azadirachtin to be comprised of two main (azadirachtin A & B) and two minor (azadirachtin C & D) analogs (Rembold *et al.*, 1986). However, extensive studies utilising mass spectrometry (MS), NMR, high-performance liquid chromatography (HPLC) and more recently multilayer countercurrent chromatography (MLCCC) have shown that azadirachtin is actually composed of at least 14 distinct congeners, some of which have modified ester groups attached to C-1 and C-3. These have been termed azadirachtins A-N (Govindachari *et al.*, 1992a, 1992b; Kraus, 2002 and Luo *et al.*, 1999). Of these, azadirachtin A ( $\text{C}_{35}\text{H}_{44}\text{O}_{16}$ , Molecular Ratio 720 (figure 1.9.B.)) is the major component (approximately 80%) of the total azadirachtin. The isomer 3-tigloylazadirachtol (azadirachtin B) is present at concentrations up to 15% of that of azadirachtin. The azadirachtins (C – N) occur at much lower concentrations (Mordue (Luntz), 1997; Mulla & Su 1999 and Rembold, 1989).

Azadirachtin has been described as being distinct from the other limonoids present in neem, in that the molecule can be characterised by the presence of a 1,3-dioxygenated A-ring and, at the opposing end of the structure, a dihydrofuran system (Govindachari, 1992).

Durand-Réville *et al.*, (2001) and Ley *et al.*, (1989b) have studied the functional groups of azadirachtin in detail. Their work has shown that azadirachtin is one of the most highly oxidised limonoids known, containing 16 chiral centres as well as a plethora of oxygen functionality. The azadirachtin molecule also comprises an enol ether, an acetal, a hemiacetal and a tetra-substituted oxirane as well as a variety of carboxylic esters. In addition, both secondary and tertiary hydroxyl groups and a tetrahydrofuran moiety are also present.

### **1.9. Azadirachtin related limonoids**

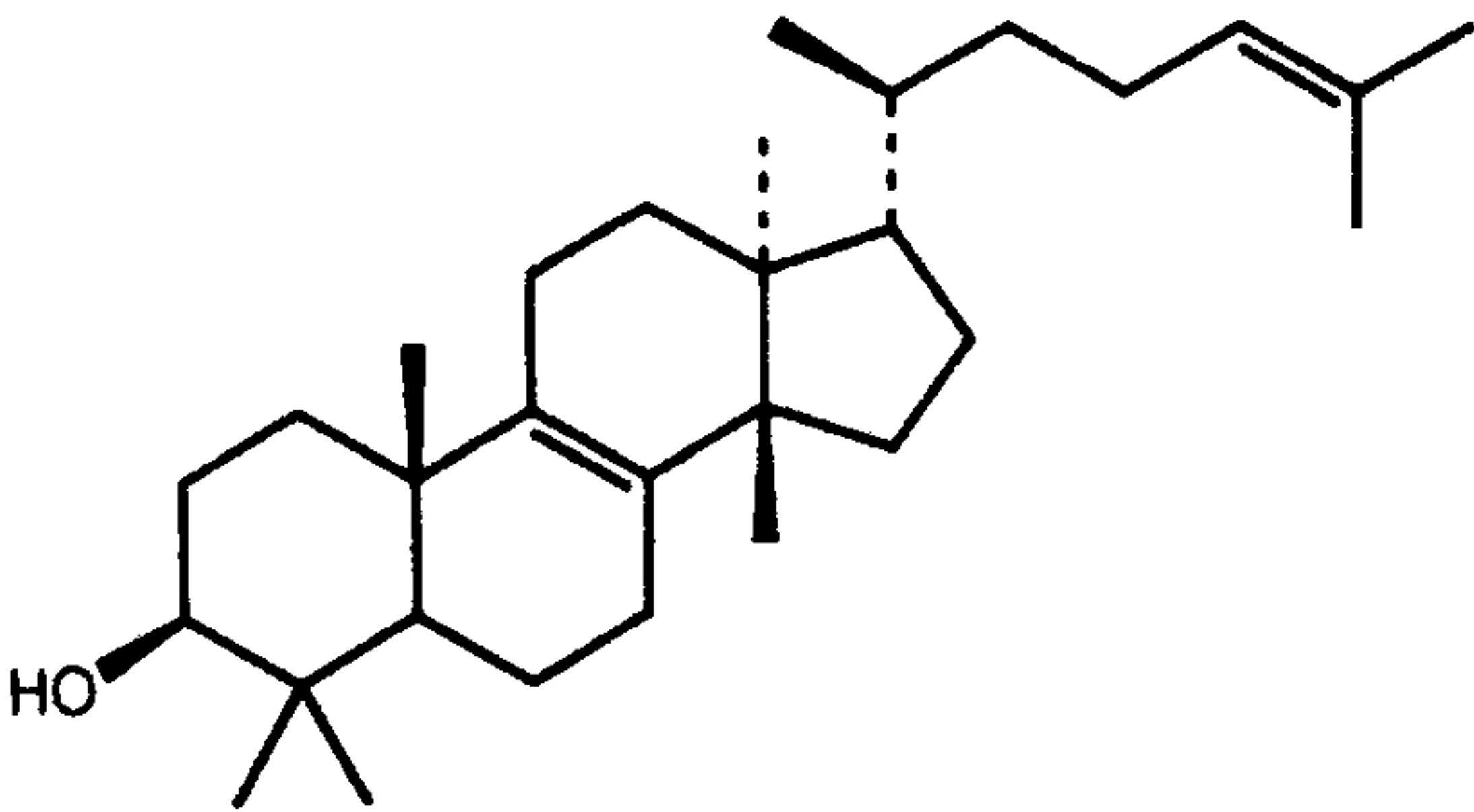
Although azadirachtin is regarded as the principal active constituent in neem extracts, it is accompanied by a number of related compounds that are of considerable interest from a structural and biological point of view (Morgan & Jarvis, 2001). Of these, there are several limonoids in which the A, C or D rings have been opened by oxidation (Jones *et al.*, 1988). However, it is the group which azadirachtin belongs to, the C-seco limonoids, which are believed to represent the most important source of potential insecticides (Kraus, 2002).

Siddiqui (1942) was the first to isolate a member of this group from seed oil. This limonoid was termed nimbin ( $C_{30}H_{36}O_9$ , Molecular Ratio 540 (figure 1.9.C.)) and

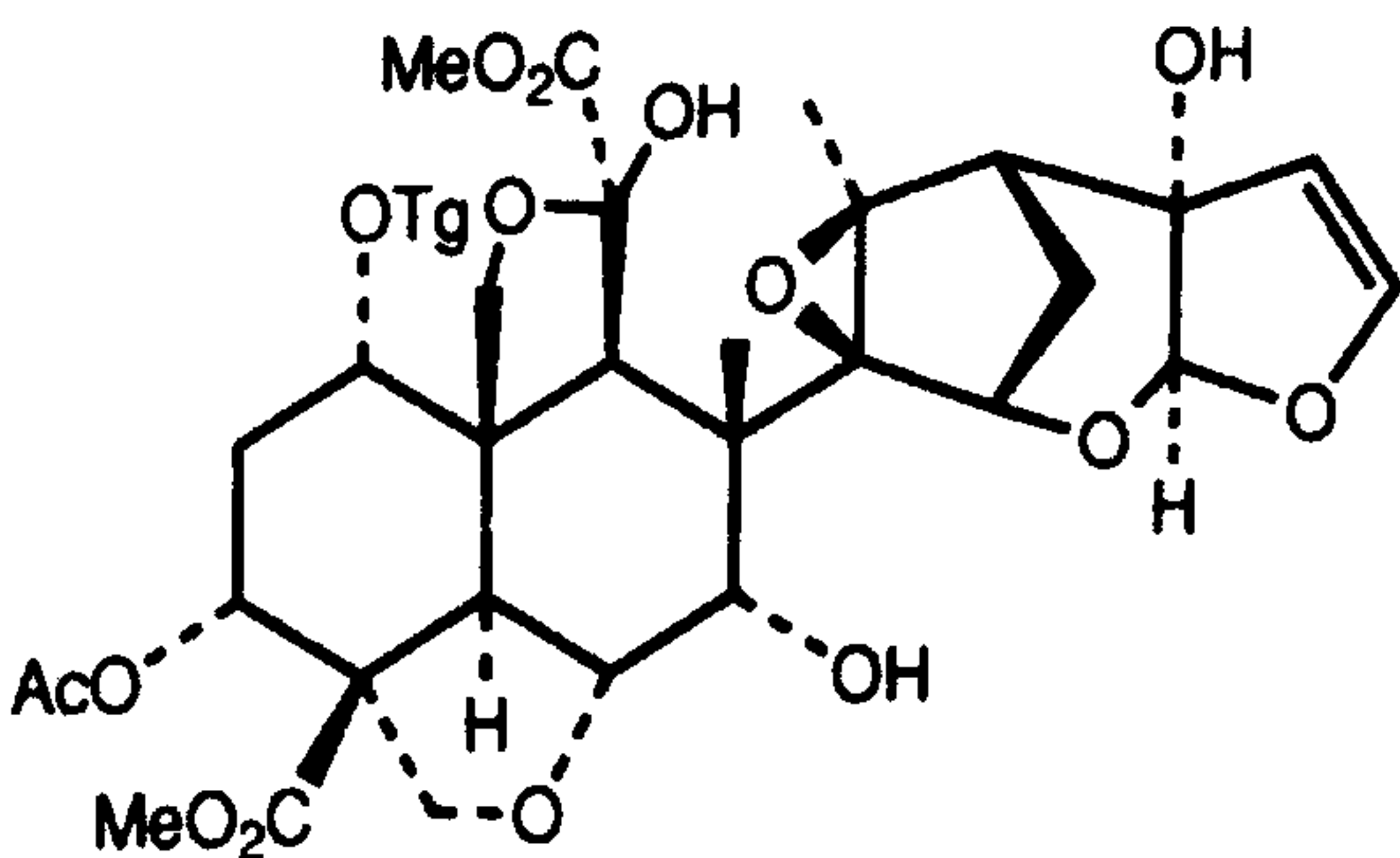
like azadirachtin, it was a number of years before its structure was correctly elucidated (Jones *et al.*, 1988). In follow-up work conducted by Henderson *et al.*, (1968) a second structurally similar member of the group was isolated and identified from seed oil. This was termed salannin (C<sub>34</sub> H<sub>44</sub> O<sub>9</sub>, Molecular Ratio 596 (figure 1.9.D.)) and was characterised by the presence of two oxygen bridges at C-6/28 and C-7/14 (Kraus, 2002).

Nimbin and salannin are two of the most abundant limonoids present within neem seeds, accounting for approximately 30% and 6% of the total limonoid quantity respectively (Morgan & Jarvis, 2001). However, a considerable number of other, less abundant compounds have been isolated from various parts of the tree and their structures elucidated. Some of these are regarded as analogues or derivatives of either nimbin or salannin and include nimbolide, nimbinol, 6-deacetylnimbin, 28-deoxonimbolide, salannol, 3-deacetylsalannin and salannolacetate (Bokel *et al.*, 1990 and Kraus, 2002). In addition, accompanying these limonoids are a number of distinct groups that have been isolated and studied for biological activity. These include protolimonoids, pentanortriterpenoids, hexanortriterpenoids and nontriterpenoidal constituents (Koul *et al.*, 1990). However, research into these groups remains limited and it is not clear what role, if any, many of these substances will play in the future development of neem insecticides. Nevertheless, the search for new and as yet unidentified components from the neem tree continues.

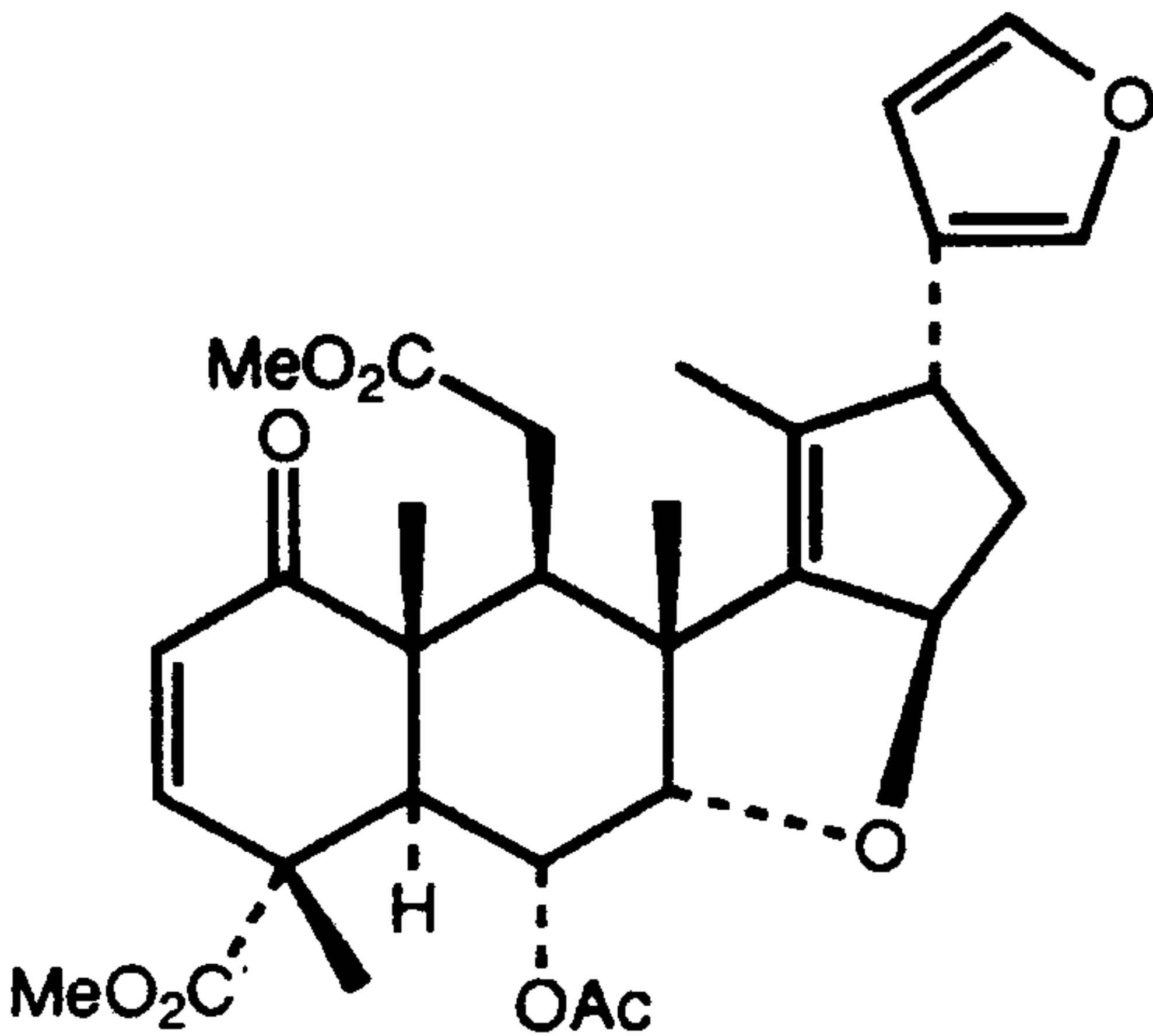
Figure 1.9. The major triterpenoids of neem and their parent steroidal intermediate, tirucallol.



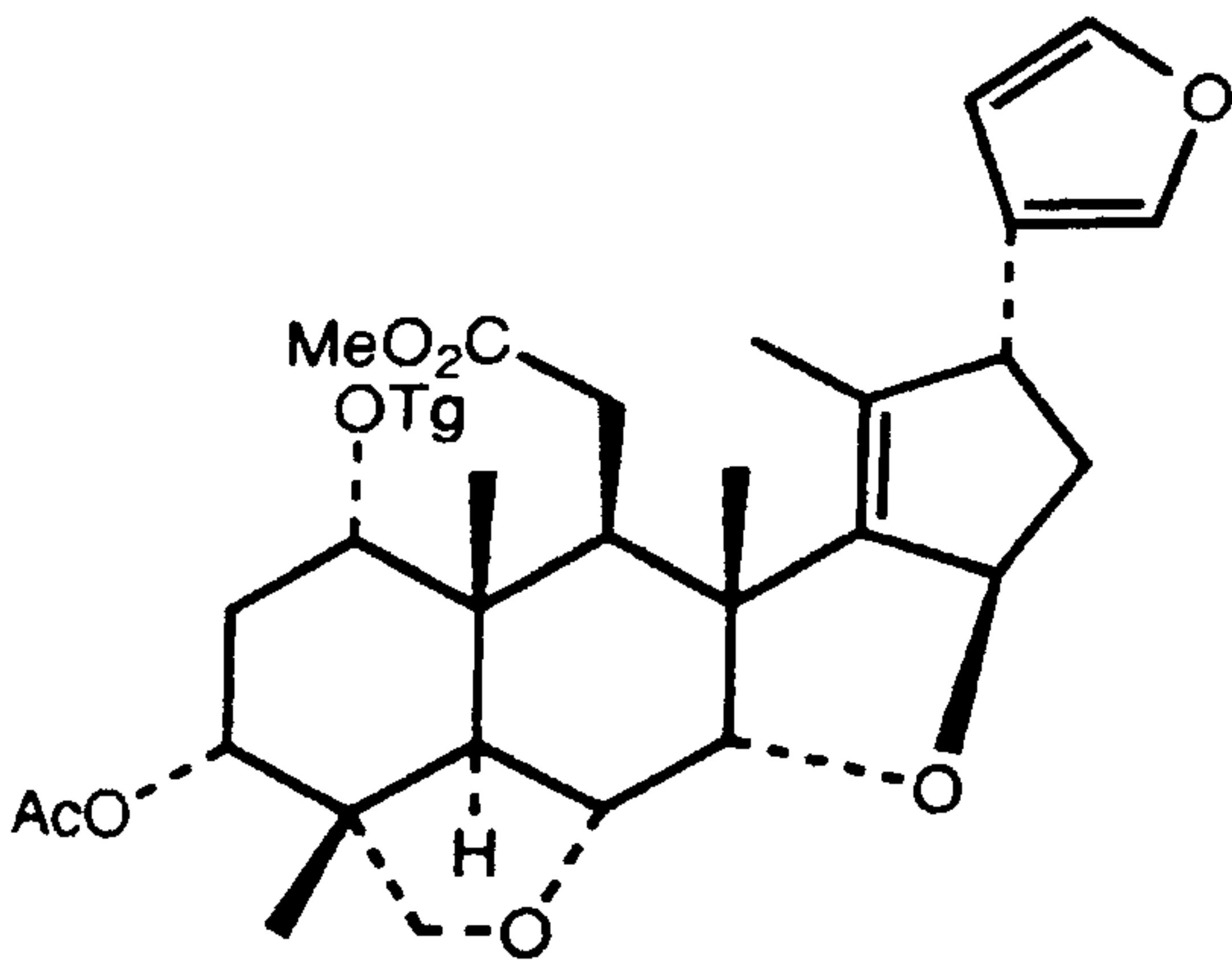
(A) Tirucallol



(B) Azadirachtin A



(C) Nimbin



(D) Salannin



### **1.10. Biological activity of azadirachtin and its related terpenoids on insects**

Studies into the biological activity of neem extracts far eclipse the work that has been conducted on identifying the chemical characteristics of isolated compounds. Most of these investigations have been performed with either azadirachtin or cruder neem extract such as neem seed oil. Only relatively few studies have investigated the insecticidal potential of isolated components related to azadirachtin, such as nimbin and salannin, and it is generally regarded that these contribute less to the insecticidal properties of the neem tree. In this respect, Kumar & Parmar (1996) correlated the biological activity of neem to the content of azadirachtin and to a lesser extent salannin, while simultaneously showing that nimbin did not contribute to observed insecticidal action.

It has been observed in numerous studies that the insecticidal performance of neem products against most insects is not as dramatic as that of synthetic insecticides and for equivalent effectiveness, considerably higher doses are required (Parmar, 1986). However, in comparison with other biological insecticides, neem products often compare more favourably. Kirsh (1986) showed that neem extracts could be more effective in controlling certain pests of tobacco and cabbage than products based on *Bacillus thuringiensis*. Similarly, Lowery & Isman (1994b) reported that neem seed oil and neem seed extract were as effective, or better than, the botanical insecticide pyrethrum for the control of aphids under certain field conditions.

Numerous reports (for example Bidmon *et al.*, 1986; Blaney & Simmonds, 1990; Jaglan *et al.*, 1997; Mordue (Luntz) & Blackwell, 1993; Naqvi, 1986; Nisbet, 1992

and Schluter, 1986) have clearly shown that neem components are capable of producing multiple effects in insects, including antifeedancy, growth regulation, fecundity suppression and sterilization, oviposition repellence and changes in biological fitness. As would be expected, clear differences have been shown to exist in the magnitude of these effects, depending on variables such as concentration (Jaglan *et al.*, 1997), formulation of the active principle (Stark & Walter, 1995b), application method (Arpaia & van Loon, 1993) and target species (Maredia *et al.*, 1992). Lowery & Isman (1994a) also suggested the possibility of intraspecies variability in the susceptibility of insects from different biotypes to neem.

#### 1.10.1. Antifeedancy

There are extensive reports detailing the strong antifeedant effects brought on by exposure to neem derivatives. Chemicals described as antifeedants are not necessarily toxic to insects but can prevent or reduce feeding when perceived. Consequently, insect growth, development, survival, and reproduction are adversely affected (Saxena *et al.*, 1988). Various orders of insects have been recorded being sensitive to neem in this respect. Examples include Lepidoptera, Coleoptera, Hemiptera, Isoptera, Diptera, and Orthoptera. Azadirachtin itself has been described as a classic example of a natural plant defence chemical affecting feeding (Mordue (Luntz) & Blackwell, 1993; Saxena, 1986 and Saxena *et al.*, 1988).

The terpenoid has been shown to produce two distinct antifeedant responses in insects, which have been reviewed by Mordue (Luntz) (1997). Primary (gustatory) antifeedancy describes the affects where azadirachtin is perceived at the

chemoreceptor level by taste receptors on the insect's mouthparts and feet. Behavioural avoidance is then initiated following the modification of sensory inputs by azadirachtin, of deterrent cells that inhibit the sugar (phagostimulatory) cells in these receptors (Schoonhoven, 1982 and Simmonds & Blaney, 1983). Secondary types of antifeedant responses are described as "anorexia" and are initiated following the onset of physiological toxic affects arising from azadirachtin uptake or ingestion. Blaney *et al.*, (1990) showed that insects belonging to the same order could exhibit both of these responses when they recorded the reactions of several species of lepidopterous larvae to deposits of azadirachtin and its related limonoids.

Arnason *et al.*, (1985) presented interesting observations on the development of antifeedant behaviour, when they compared the sensitivity of responses of the European corn borer, *Ostrinia nubilalis* to those of the fall army worm, *Spodoptera frugiperda* collected from African locations. This work showed that *S. frugiperda* was approximately 10 times more sensitive to azadirachtin deposits in antifeedant studies. This difference was attributed to a behavioural adaptation of the African insects to avoid the toxic effects of limonoids present in plants, which are common throughout their region of collection. In contrast, because limonoid-containing plants are restricted in distribution to tropical areas, this sensitivity had not evolved in *O. nubilalis* where the selection pressure was absent.

In terms of insect pest control, Schmutterer (1986) contributed important findings regarding the sensitivity of azadirachtin-induced antifeedancy, during studies on the feeding behaviour of the Colorado potato beetle, *Leptinotarsa decemlineata*. These were that the level of antifeedancy might be negatively correlated to temperature. The

evidence for this was greater levels of food rejection at 22°C compared to 27°C. Additionally, Lowery & Isman (1994a) who studied the consequences of antifeedant effects in insects, concluded that feeding deterrence does not contribute significantly to the control of aphid populations. They showed that the antifeedant affect within the strawberry aphid, *Chaetosiphon fragaefolii* did not persist after 24 hours. Alternatively, it was proposed that control would result from the prevention of nymphal moulting and adult reproduction.

#### 1.10.2. Effect on metamorphosis/growth regulation & biological fitness costs

Treatment of insects by injection, oral ingestion, or topical application of azadirachtin has been shown to induce growth inhibition, prolonged development periods, disruption of biological rhythms, malformation, immunodepression, enzyme inhibition, poor nutrient adsorption, reduced life span and mortality (Ascher, 1997; Mulla & Su, 1999; Parmar, 1986 and Wilps 1986). Ultimately, these represent biological fitness costs that are being imposed in treated individuals. These effects have been expressed in Coleoptera, Orthoptera, Hemiptera, Heteroptera, Lepidoptera and Diptera (Ascher, 1993 and Jacobson, 1986).

Prabhaker *et al.*, (1986) showed that neem extracts function in a manner similar to that of other insect growth regulators. Rembold *et al.*, (1986) and Ascher (1993) studied the responses of insects to neem in this respect. During these investigations they were able to demonstrate that the major action of azadirachtin was through its ability to modify or suppress hemolymph ecdysteroid and juvenile hormone titers by



inhibiting the release of morphogenetic peptide prothoracicotropic hormone (PTTH) and allatotropins from the brain-corpus cardiacum complex.

There have been numerous studies investigating the various ways in which these growth-regulating effects are expressed in different potential targets. Work in this area clearly shows a species-dependent sensitivity in regard to the onset and symptoms of poisoning. Redfern *et al.*, (1984) observed that the last instars of the large milkweed bug, *Oncopeltus fasciatus* having been treated topically with 0.1µg azadirachtin, failed to moult and showed split cuticles. Similarly, Koul (1984) reported that azadirachtin caused wing deformities, nonplastication of wing lobes, development of wingless adults and larval mortality following application to various stages of the red cotton bug, *Dysdercus Koenigii*.

Bidmon *et al.*, (1986) studied the range of fitness costs expressed throughout the life cycle of the blowfly, *Phormia terrae-novae*. Following the treatment of larvae, several detrimental effects on morphology were recorded. These included a prolonged third instar, a reduction in the number of pupae formed, weight loss and malformations in both larvae and pupae, and mortality at higher doses. Flies that emerged from pupae treated with azadirachtin were smaller and showed deformations in wings, legs, proboscis and abdomen. It was concluded that these individuals would suffer severely in terms of reproductive potential and survival rates in comparison to healthy populations.

Dorn *et al.*, (1986) made important observations regarding the biological fitness cost expressed in *O. fasciatus*, when they discovered a variation in sensitivity to

azadirachtin between male and female individuals. This research showed that following the treatment of female insects with 0.25µg azadirachtin, longevity was markedly reduced (to 11 days or less). However, in contrast, concentrations of 1µg or higher were required to reduce male longevity to a similar extent.

A final important consideration regarding the growth regulating ability of neem was highlighted by Timmins & Reynolds (1992), who studied the effects of azadirachtin on the tobacco hornworm, *Manduca sexta*. Their work was to show that growth regulation could occur independently of the antifeedant effect. Inhibition in this instance was associated with increased costs of growth. These increased costs were shown to result from a decrease in the efficiency of dietary nitrogen utilisation, which was linked to a reduction in the activity of the midgut trypsin.

#### 1.10.3. Fecundity, egg-sterility and reproductive effects

The potential of neem in reducing the fecundity of insect pest populations is of considerable interest to the scientific community and has significant consequences for insect control using these products. Numerous investigations have elucidated the mode of action of azadirachtin in this respect. These studies have shown that because ecdysteroid is one of the hormones regulating vitellogenesis, and because azadirachtin can modify hemolymph ecdysteroid by inhibiting the release of PTTH and allatotropins from the brain-corpus cardiacum complex, adverse effects on ovarian development, fecundity and fertility can occur. These effects have been recorded in Orthoptera, Hemiptera, Heteroptera, Coleoptera, Lepidoptera, Diptera and Hymenoptera (Ascher, 1993; Isman, 1990 and Simmonds *et al.*, 1990).



To assess the potential of an effective sterilant for pest control, the speed and the duration of the effect should be known. Experiments conducted by Schmutterer (1986) on the reproductive period of *L. decemlineata* showed that neem extracts act very quickly (within 24 hours) and that their fecundity-reducing and sterilizing effects can persist throughout the period of reproduction. Schmutterer also noted that in *L. decemlineata*, reduced hatching rates were not a result of egg sterility but instead were mainly due to the excretion of a sticky orange-yellow liquid by treated females. This excretion was shown to be a product of dissolved eggs in the ovarioles, which produced a covering layer that resulted in embryo suffocation.

In addition, Wilps (1986) proposed a further reason for reduced hatching rates in some insects treated with azadirachtin. It was demonstrated that by adding azadirachtin to the protein diet on which blowfly individuals were raised, eggs exhibited a variety of defects and were more prone to infection by fungi. These findings were in agreement with results presented by Ascher (1981).

Shimizu (1988) discovered an additional reproductive effect caused by azadirachtin. Research conducted by this author showed that spermatogenesis could be disrupted by azadirachtin treatments. This resulted in hatching rates being reduced through a reduction in egg fertility.

Besides physiological alterations in target insects, azadirachtin has been shown to modify the reproductive behaviour of both males and females in mating and in response, while having no apparent physical influence. Schmutterer (1990) illustrated this effect during research involving the brown rice planthopper, *Nilaparvata lugens*.

Female individuals that had been topically treated with 2.5µg or 5µg neem oil or sprayed with a 3% neem oil solution, failed to produce normal courtship signals. At higher concentrations most females did not emit signals, which resulted in males being unable to locate them.

Dorn *et al.*, (1986) linked these reproductive behavioural responses to the physiological consequences of azadirachtin exposure. The results of this investigation were to show that despite the initiation of mating behaviour, doses of azadirachtin as low as 0.125µg, injected into the abdomen of individuals, induced impotence in male *O. fasciatus*, which prevented copulation.

#### 1.10.4. Orientation repellence

Numerous studies (for example Akou-Edi, 1983; Coudriet *et al.*, 1985 and Saxena *et al.*, 1981) have highlighted the repellent activity of neem extracts against a wide range of insect pests. Dethier (1956) defined repellence as the orientated movement away from a stimulus source and it this form of behaviour that is often regarded as the first barrier against insect attack (Saxena, 2002). However, following the application of neem, the magnitude and consequences of these repellent effects are often unclear and in certain cases the application technique and duration of exposure can have a significant impact on repellent activity.

Grace & Yates (1992) presented evidence on the repellent activity of the neem insecticide Margosan-O towards the formosan subterranean termite, *Coptotermes formosanus*. This research correlated the duration of exposure to repellent behaviour

and showed that repellence did not occur from short (20-minute) exposures. Only over relatively long periods (10 days) did individuals begin to avoid contact and orientate away from treated areas.

Hunter & Ullman (1992) illustrated a second important consideration regarding repellent behaviour. They studied the transmission of zucchini yellow mosaic virus by the pea aphid, *Acyrtosiphon pisum* following exposure to the neem insecticide RD-Repelin. This work showed significant repellence that included both pre- and post probing components along with other behavioural observations, which suggested that aphids could detect the insecticide through olfactory stimuli. Importantly, these results showed that following a disturbance of settling behaviour, aphids would tend to initiate a wandering response. As a consequence of this, it was concluded that the spread of the virus from infection sources could be accelerated, as aphids disperse in search of acceptable hosts.

#### 1.10.5. Oviposition repellence

The success of neem products at producing oviposition repellence is often unclear because of conflicting results between laboratory and field investigations. However, a number of responses have been recorded following the treatment of oviposition sites with azadirachtin. These include oviposition repellence, deterrence, or inhibition, all of which have been shown to occur in Homoptera, Coleoptera, Lepidoptera and Diptera (Jacobson, 1986; Schmutterer, 1990 and Wilps, 1986).

Parmar (1986) reviewed the literature concerning oviposition repellence and concluded that high concentrations would be needed for this to be achieved. Research by Liang *et al.*, (2003) supports this statement. This author noted that relatively high concentrations were required to inhibit egg laying in the diamondback moth, *Plutella xylostella* following the treatment of oviposition sites with three neem-based insecticides (Agroneem, Ecozin and Neemix). This work demonstrated that the three commercial formulations did not produce significant inhibition, despite considerable mortality and antifeedant effects being achieved at the concentrations tested. Liang *et al.*, concluded that this effect could be attributed to the cabbage leaves producing a stronger volatile that overshadowed the odour of neem insecticides.

Klemm & Schmutterer (1993) also studied the reactions of *P. xylostella* to treatments of oviposition sites. Through this research the sensitivity and variability of response was highlighted. In this case, data from both field and laboratory trials showed that in some instances, oviposition could be encouraged. This statement was based on the observation that *P. xylostella* would oviposit more frequently on cabbage leaves following treatment with neem seed kernel water extracts.

### **1.11. Problems associated with neem**

In reviewing the literature, the problems associated with neem use are often unclear and in some cases these can be seen as dilemmas that face botanical pesticide use in general. This is partly due to inconsistencies in the material used and in the interpretation of data, but also because of a lack of experimental work, particularly



under field conditions. Consequently, further work is required to clarify some of these topical issues.

#### 1.11.1. Delayed effects of insecticidal action

A potential barrier to the wide-scale use of neem-based products is concerned with the delayed effect of such materials, especially when this is compared to the almost immediate action of synthetic neurotoxins. This delay in the onset of mortality will permit many insect pests to continue to feed on treated material following application. In some markets, particularly high value ones, this will not be acceptable. However, in such instances Schmutterer (1990) stated that the quantity of food consumed would be markedly reduced as a result of the “secondary” antifeedant effect (see section 1.10.1.).

Conversely, Liang *et al.*, (2003) noted that following the treatment of larger *P. xylostella* larvae on cabbage, considerable foliar damage was still possible during this period prior to mortality. It was concluded from this work that neem-based insecticides should be applied as early as possible when the insects are egg, neonates, or second instar, in order to prevent significant foliar damage.

#### 1.11.2. Variable and limited field performance

The field performance of neem products may be more variable depending on environmental conditions than that of their synthetic counterparts. Consequently,



prevailing weather may play an important role in determining the success of a neem application.

Caboni *et al.*, (2002) conducted field trials to study the persistence of efficacy of azadirachtin using both pure compound and commercial formulations. This research showed that the loss of efficacy was unrelated to either evaporation or thermodegradation, but instead was attributed to photodegradation. Importantly, this work also showed that azadirachtin's breakdown occurred approximately 5 times faster in commercial formulations than when compared to the pure compound. It was concluded that the formulation additives acted as catalysts for accelerated breakdown.

In similar studies investigating the limited persistence of azadirachtin formulations, Sundaram & Curry (1996) found that the use of selective UV absorbers could stabilise azadirachtin in the presence of strong ultra-violet light. One pre-requisite of the UV absorber was shown to be that its UV spectrum and  $\lambda$ -max must be similar to those of azadirachtin. Sundaram & Curry concluded that stabilisation could be achieved by either transferring energy from azadirachtin to the UV absorber and/or through competitive absorption of UV photons by the absorber. However, these conclusions were based on laboratory studies and therefore their relevance to field conditions remains unclear.

Another important consideration regarding the field performance of neem, relates to the ability of target insects to differentiate between treated and untreated plant surfaces (i.e. resistance through positive avoidance of residues). Strang (personal communication) suggested that some insects possess receptors which recognise neem

terpenoids with great specificity and high affinity. In this respect, Schmutterer (1990) stated that neem pesticides should be applied either at high volume or by using techniques/technologies that may guarantee an even distribution of the active material on plant surfaces. This would therefore ensure that a pest insect would not preferentially feed on untreated plant parts.

Muda & Cribb (1999) studied the importance of achieving a uniform distribution of neem products in relation to insect pest control. This research studied the effects of uneven applications to wheat grain, on the reproduction and feeding behaviour in the lesser grain borer, *Rhyzopertha dominica*. In contrast to Schmutterer's statements, these results showed that the antifeedant effect of azadirachtin was unaffected by unevenness of treatment, provided that 50% of the grain was treated. Similarly, treating 10% of the grain produced the same effect on reproduction as treating 100%. In relation to field applications, these results were regarded as of significant importance because uniform distributions of insecticides may be difficult, or impossible to achieve. Additionally, these results were related to the potential development of resistance. From this research it was concluded that because a uniform coverage of material is not required to produce acceptable control, the possibility of resistance development associated with positive avoidance of treated surfaces is reduced.

### 1.11.3. Resistance

Relatively little research has been conducted to determine the potential of insects developing resistance to components of neem following repeated exposure.

Nevertheless, work to date has reached some important conclusions regarding the likelihood of this occurring towards neem products.

Völlinger (1986) compared the possible development of resistance in *P. xylostella* towards neem seed kernel extracts and to the synthetic pyrethroid, deltamethrin. This research demonstrated that after 42 generations, larvae showed no signs of resistance in feeding and fecundity tests towards the neem extract. However, in contrast, significant (x35) resistance towards deltamethrin was recorded.

Feng & Isman (1995) conducted similar trials to investigate the selection for resistance to azadirachtin in the green peach aphid, *Myzus persicae*. They compared resistance development resulting from pure azadirachtin to that arising from a refined neem seed extract over 40 generations. Similar to Völlinger's observations, *M. persicae* did not exhibit any signs of resistance towards the neem seed extract. However, following repeated exposure towards purified azadirachtin, at equivalent concentrations, a moderate (x9) level of resistance developed. It was concluded from this work that a mixture of active constituents (as in the neem seed extract) might diffuse the selection process, when compared to that expected from a single active ingredient. Two possible explanations for this effect were proposed. Firstly, that the various terpenoids such as nimbin, salannin and azadirachtin may have different sites or modes of action, and secondly, that these constituents may play some role in inhibiting the enzymes which may be responsible for azadirachtin breakdown.

However, both of these studies were based on selection for resistance in laboratory studies and were not supported by field trials, where selection pressures may be

realistically higher because concentrations applied tend to be greater. Consequently, additional studies are necessary to determine the likely-hood of resistance development under more realistic conditions.

#### 1.11.4. Commercial production

A considerable technical limitation concerning azadirachtin use is in regard to its commercial production. Even though extensive efforts have been made to synthesise the molecule, success is still perceived by many as a long way off due to structural complexity (Mulla & Su, 1999). Systematic studies conducted by Ley *et al.*, (for example 1988, 1989a, 1989b, 1991) have come closest to producing a synthetic version of azadirachtin. However, despite the success of this research in synthesising both the decalin and hydroxyfuranacetal fragments, attempts to couple the two portions have been unsuccessful due to the extreme steric congestion around the C18-C14 bond (Durand- Réville *et al.*, 2002).

Consequently, the manufacturing of neem-based products will for the foreseeable future rely on natural material obtained from the tree. However, this in itself is problematic due to the low abundance and variability of azadirachtin in the plant, (0.02%-0.35% w/w in seed kernels) which makes it difficult to obtain pure extract in sufficient quantities for crop protection (Ley & Toogood, 1990 and van Beek & de Groot, 1986). Currently, there are approximately 70 countries that are raising neem plantations for commercial interests (Sidhu *et al.*, 2003). Therefore, it is important that the variability of neem is understood so that standardised neem preparations can be produced that exhibit comparable biological activities.



In an attempt to identify the causal factor(s) behind this variability, Ermel *et al.*, (1983) studied the variation of azadirachtin content between neem trees of African and Asian origin. Comparisons from this research showed that trees grown in India and Togo yielded greater amounts of azadirachtin than trees found in the Sudan. It was concluded that environmental conditions might influence azadirachtin content. Research by Singh (1986) seems to support these conclusions through a comparison of azadirachtin yield from neem trees of different ecotypes. This research suggested that variations in the quantity and quality of neem extracts could be expected due to different ecological conditions under which the plant grows. However, when Gupta & Prabhu (1997) studied variations of azadirachtin content throughout the Rajasthan state, no correlation could be made between azadirachtin content and climatic conditions within the region of collection.

In an attempt to clarify variability, Sidhu *et al.*, (2003) studied the differences in neem with respect to azadirachtin content throughout a number of provinces of India. This work showed that large variations existed between trees. However, these studies revealed that climatic factors such as rainfall, humidity, or temperature did not influence azadirachtin content. Therefore, an alternative explanation to variability was proposed. From this work, Sidhu *et al.*, suggested that genetic differences were more likely to be responsible for variations between individual trees.



#### 1.11.5. Off-target effects

In addition to these technical limitations, other problems that relate to neem are concerned with adverse effects on non-target organisms including beneficial insects, aquatic invertebrates and humans.

Compared with synthetic insecticides, neem derivatives possess as a rule, weak to moderate or no contact activity against insect pests and therefore require ingestion to exert their toxic effects (Saxena, 2002). Consequently, neem-based products are generally regarded as being safe towards beneficial organisms such as honeybees and natural enemies and their compatibility within many IPM programmes depends on this feature. Tedeschi *et al.*, (2001) investigated this compatibility by studying the side effects produced by three neem formulations on the myrid predator, *Macrolophus caliginosus*. From this work they concluded that neem formulations were safe towards beneficial predators because of the short persistence of formulations (<5days). Interestingly, this conclusion was reached despite fecundity suppression and significant mortality being observed following treatments.

However, when Qi *et al.*, (2001) studied the indirect effects of neem exposure towards the insect predators, *Harmonia conformis* and *Mallada signatus*, following consumption of neem-fed prey (*Helicoverpa armigera*) an alternative conclusion was reached. This work showed that feeding on neem-fed prey extended larval stages, prolonged pupation and produced significant mortality in both beneficial species. Consequently, it was concluded that these indirect effects would have to be taken into

account in any IPM program that relies on neem applications in concert with persistent natural enemy populations.

Neem applications have been shown to not only impact on beneficial insects but non-target aquatic invertebrates are also at risk. Kreutzweiser *et al.*, (2002) evaluated community-level disruptions among zooplankton following neem applications. This work showed that zooplankton communities could be at risk of adverse affects from neem-based insecticide spraying at both high and low concentrations. It was concluded therefore, that mitigating measures such as buffer zones around standing water bodies and improved application technologies to reduce off-target spray drift should be implemented during neem applications.

#### 1.11.6. Safety towards mammals

The safety of neem, and more specifically azadirachtin, towards mammals is not well documented, despite the terpenoid being generally considered as safe in this respect.

Through a subchronic toxicity assessment in rats, Raizada *et al.*, (2001) showed that azadirachtin has a LD<sub>50</sub> of more than 5,000mg/kg, comparing extremely favourably to those of other botanical insecticides (e.g. nicotine = 50mg/kg, pyrethrum = 750mg/kg) and synthetic insecticides (e.g. DDT = 113mg/kg, cyfluthrin = 250mg/kg) (PAN UK, 2001). Consequently, this feature has been a major driving force behind research to date. Investigations such as those by Salehzadeh *et al.*, (2002) are beginning to show that azadirachtin has differential toxicity towards insect and mammalian cells.

However, neem is not completely risk-free and falls into class III (slightly hazardous) of the World Health Organisation toxicity rating (PAN UK, 2001). Sinniah *et al.*, (1982) documented occasional fatalities in children following oral ingestion of large doses of neem oil for minor ailments. Symptoms of poisoning included vomiting, drowsiness, metabolic acidosis, and encephalopathy. The characteristics of poisoning in this case were described as like Reye's syndrome. Neem has also been shown to cause liver enlargements, respiratory difficulties, seizures, diarrhoea, nausea, and general discomfort in humans (Jacobson, 1988).

However, it is important to keep these findings in perspective with other pesticides. Studies by Awasthy *et al.*, (1995) showed that despite producing a number of chromosomal abnormalities in bone marrow cells of mice, neem extracts exhibited considerably less damage than synthetic pesticides.

### **1.12. The formulation of neem as an insecticide**

Neem has been used in many different forms against insects. These range from simple preparations and extracts to sophisticated and specialised formulations that rival those of any synthetic insecticidal product. This adaptability and compatibility of neem is one of the many reasons as to why the botanical product has found uses in a number of different areas.

Perhaps the most basic application of neem employs the use of simply-prepared plant material. Koul *et al.*, (1990) described the age-old practice in India of storing dried neem leaves in grain or amongst clothes to repel insects.



Numerous methods however, have been employed to obtain enriched extracts from the tree which exhibit enhanced biological activity. Perhaps the simplest involves pressing the seed kernels to collect the oil. Neem seed oil has been used for some time in the developing world against insect pests. Typically the oil is used without further modification, but growing commercial interest has seen the oil being purified to remove sulphur and fatty acid components (National Research Council, 1992). This purified product may then have emulsifiers added to it to improve spraying characteristics (Hellpap & Dreyer, 1995). The insecticidal potential of neem oil formulations has now been widely studied. Investigations such as those conducted by Ketar (1986) have shown that these formulations can be highly effective as surface protectants. Consequently, neem seed oil formulations have found commercial applications against pests such as aphids, jassids, thrips, mites, and helopelties (Kulkarni *et al.*, 1999).

As an alternative to pressing the oil, simple extracts can be made in an attempt to remove the biologically active components. A pesticidal “cocktail” can be easily obtained from neem leaves or seeds when they are crushed and steeped in water, alcohol, or other polar solvent (Norten, 2000). The insecticidal activity of these extracts can be widely variable depending on precise extraction procedure. However, it is generally agreed that extracts from seeds tend to exhibit greater insecticidal effect than those from the leaves. In addition, superior biological activity is achieved through extraction with alcohol when compared to those made under aqueous conditions (Karel, 1986).

Because of increases in cost associated with further purification, neem seed oil or the alcoholic extracts of the seed kernels are used as the basis of modern commercial formulations. Typically, neem is formulated as an emulsifiable concentrate, composed of various solvents and surfactants (Kumar & Parmar, 2000). However, the constituents have also been formulated as wettable powders, liquid concentrates and as flowable concentrates (California Environmental Protection Agency, Department of Pesticide Regulation, 2003). Figure 1.12. gives examples of neem-based products including their azadirachtin content and formulation type.

Figure 1.12. Examples of neem/azadirachtin based commercial formulations (California Environmental Protection Agency, Department of Pesticide Regulation, 2003).



Product Name	Azadirachtin Content (%)	Formulation Type
Agroneem	0.15	Emulsifiable Concentrate
Amazin 3% EC	3.00	Emulsifiable Concentrate
Amvaz AZA 3% EC	3.00	Emulsifiable Concentrate
AZA – Direct	1.20	Emulsifiable Concentrate
Azatin 4.5 WP	4.50	Wettable Powder
Azatin XL Plus	3.00	Emulsifiable Concentrate
Azatin XL Biological Insecticide	3.00	Aqueous Concentrate
Azatrol EC Insecticide	1.20	Aqueous Concentrate
Ecozin 3% EC	3.00	Emulsifiable Concentrate
Ecoside	0.15	Emulsifiable Concentrate
Fortune AZA 3% EC	3.00	Emulsifiable Concentrate
Fortune AZA Technical	14.00	Dust/Powder
Margosan-O Botanical Insecticide Concentrate	0.25	Aqueous Concentrate
Margosan-O	0.25	Emulsifiable Concentrate
NeemAzad 0.25 EC	0.25	Emulsifiable Concentrate
Neem – Away	0.09	Flowable Concentrate
NeemAzal – T/S 1.2% EC Insecticide	1.20	Emulsifiable Concentrate
NeemAzal Technical	37.00	Dust/Powder
Neemix 4.5 Botanical Agricultural Insecticide	4.50	Emulsifiable Concentrate
Neemix Botanical Agricultural Insecticide	0.25	Emulsifiable Concentrate
Turplex Biological Insecticide	3.00	Emulsifiable Concentrate
Safer Brand Bioneem Multi-purpose Insecticide and Repellent Concentrate	0.09	Flowable Concentrate
Safer Brand Bioneem Multi-purpose Insecticide and Repellent Concentrate	0.09	Solution/Liquid (Ready-to-use)
Ornazin 3% EC Botanical Insecticide	3.00	Flowable Concentrate
Ornazin 3% EC	3.00	Emulsifiable Concentrate

### **1.13. Project aims**

The previous sections of this introduction reveal that even though azadirachtin does represent a commercially viable insecticide, there has been little research into optimising the way in which the limonoid is delivered to the pest. As shown by figure 1.12., foliar applications using liquid sprays dominate the type of formulation available. However, on leaf surfaces azadirachtin exhibits poor insecticidal contact activity and a rapid degradation by photolysis. Therefore, opportunities exist for the introduction of alternative strategies, which may improve the environmental and biological efficiency of applications. In this respect, the aim of this project was to develop azadirachtin as an insecticide that is applied to the soil, using a granular formulation, for root uptake and subsequent systemic plant protection.

To achieve this goal, a number of studies would be required. The objective of these would be to:

1. Develop techniques to rapidly purify and quantify azadirachtin. The purified material would be required for chemical and biochemical investigations. The method for quantification may provide a rapid means to assess azadirachtin within crude neem seed extracts and during environmental fate studies.
2. Investigate the options for producing suitable granular formulations and assess these in terms of their stability and release characteristics. Specifically, a controlled, slow-release effect would be sought from the formulation.
3. Examine the physical/chemical suitability of azadirachtin for use as a soil applied, systemic insecticide. In particular, the mobility and stability of azadirachtin within soils and plants would be considered.

## Chapter 2

### Azadirachtin Isolation and Quantitative Estimation of Neem Seed Extracts



## **2.1. Azadirachtin isolation**

### **2.1.1. Introduction**

The isolation of azadirachtin from the seeds of *Azadirachta indica* has always been regarded as difficult. This is because seeds contain large amounts of oil (approximately 40% by weight) and also because of the structural similarity and complexity of azadirachtin and its related limonoids (Strang personal communication; Turner *et al.*, 1987 and Yamaski *et al.*, 1986). Essentially, the process of isolation requires the application of a number of techniques, such as solvent extraction, adsorption chromatography and solvent partitioning methods (Hein & Hummel, 2000.).

Regardless of the specific method used, two distinct steps can be identified in the process of isolating azadirachtin from neem seed kernels. Initially, a preparative clean-up phase is employed (figure 2.1.1.). During this step triglycerides, water-soluble proteins and sugars are removed to produce a defatted, finely-powdered concentrate. This crude extract is then suitable for a refined purification. This typically involves reversed-phase HPLC, which separates azadirachtin from other limonoids present within the crude seed extract (Morgan & Jarvis, 2001; Puri, 1999; Schroeder & Nakanishi, 1987 and Turner *et al.*, 1987).

However, semi-preparative and preparative isolation by HPLC is expensive, time-consuming and yields only relatively small amounts of pure azadirachtin (Hein & Hummel, 2000 and Thejavathi *et al.*, 1995). Therefore, alternatives have been

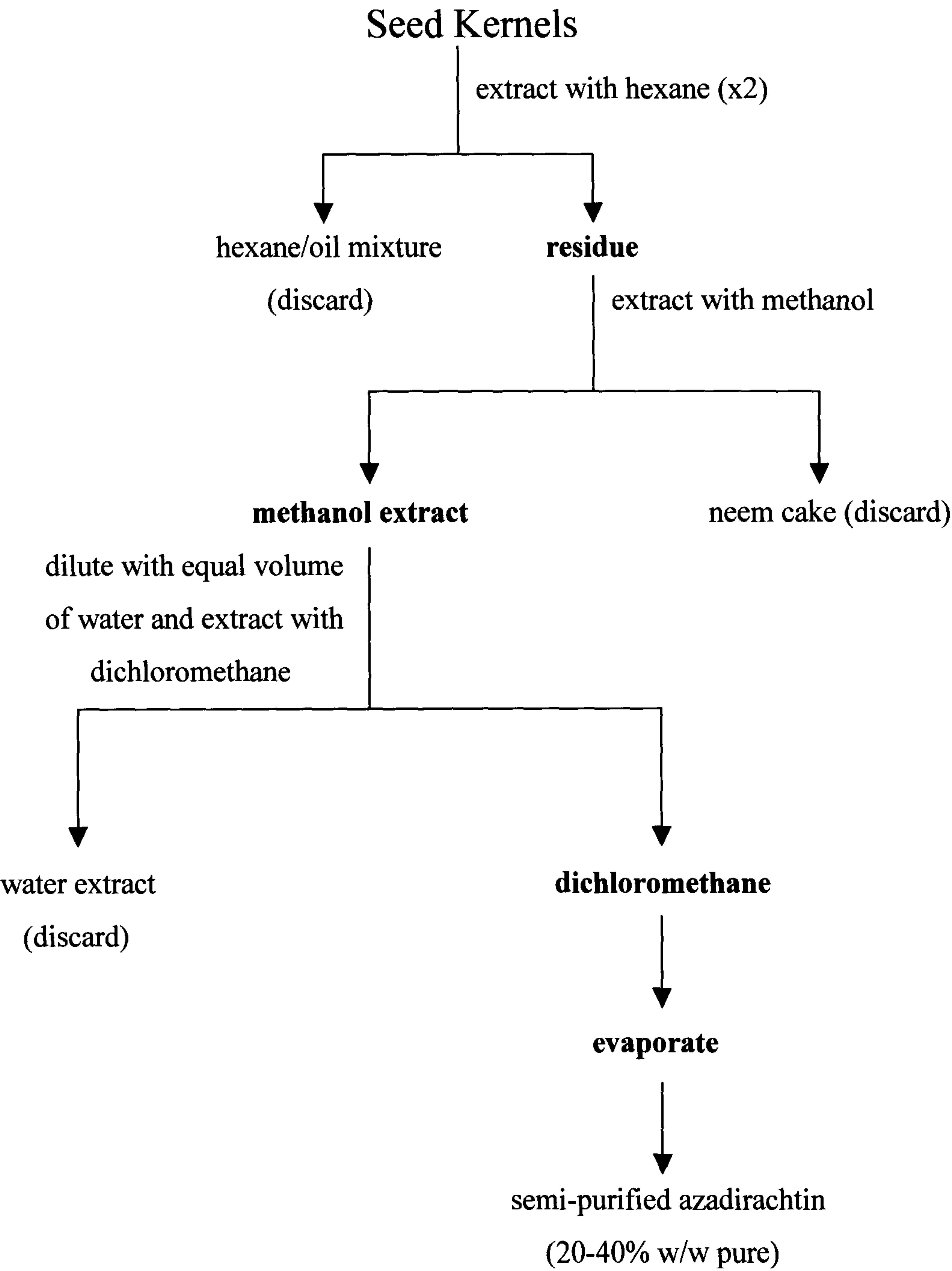
investigated and include multilayer countercurrent chromatography and supercritical fluid extraction, however these often suffer from the same disadvantages of cost, time and yield (Ambrosino *et al.*, 1999; Jarvis *et al.*, 1999 and Lou *et al.*, 1999).

### **2.1.2. Aims**

Because of the high price of pure azadirachtin (e.g. £60/mg of 95% pure material, Sigma-Aldrich, UK), purchasing suitable quantities of the limonoid for the research purposes of this project was not appropriate. Therefore, it was required that a purification procedure be developed to isolate the needed azadirachtin. The observation that azadirachtin can be obtained as a distinct peak from other neem constituents during reversed-phase HPLC, suggests the possibility of developing a simple procedure for its isolation using flash chromatography. Consequently, the aim of this work was to develop a method, based on flash chromatography and utilising non-flammable solvents (e.g. methanol and water), to rapidly isolate, large quantities of azadirachtin-A, from a semi-purified azadirachtin source (containing 35% azadirachtin-A). This method would then be used to obtain 2.5g of approximately 95% pure material for biochemical studies.

Figure 2.1.1. **Preparative clean-up phase of azadirachtin-A isolation.** Schematic representation of the method used to produce semi-purified azadirachtin, which is suitable for a refined, chromatographic purification (scheme modified from Morgan & Jarvis, 2001).





### **2.1.3. Materials and methods**

#### **2.1.3.1. General materials**

Semi-purified azadirachtin was purchased from ShriDisha Biotech, Hyderabad, India. This neem extract was used without further modification and contained approximately 35% azadirachtin-A. An authenticated azadirachtin-A standard was supplied by Dr. Robin Strang (University of Glasgow) and had a purity of greater than 95%.

### **2.1.4. Preliminary procedure for azadirachtin-A isolation**

#### **2.1.4.1. Preparation of semi-purified azadirachtin for flash chromatography**

Four grams of finely ground, semi-purified azadirachtin was dissolved in 10ml of methanol. This produced a brown solution, which was filtered using a syringe fitted with a 0.2µm syringe filter.

#### **2.1.4.2. Flash column chromatography**

A C18 flash column (Biotage 40M; 150mm x 40mm, particle size 35µm-70µm, Av. pore size: 60Å) pressurised at 1793 mbar was reconditioned with 600ml methanol and equilibrated with methanol/water in the ratio of 45:55. Pressure was controlled through a Watts pressure regulator. The methanol/semi-purified azadirachtin solution was injected onto the top of the column using a syringe. The proceeding 3000ml of eluant was collected into 15 separate fractions of 200ml. Aliquots (20µl) of each

fraction were assessed using reversed-phase HPLC (see section 2.1.4.3.) and normal-phase TLC (see section 2.1.4.4.) to determine azadirachtin-A purity. The solvent reservoir was re-charged as needed.

#### 2.1.4.3. Analytical high performance liquid chromatography using the Dionex HPLC system

Analytical reversed-phase HPLC was conducted using one of two systems. The Dionex system is described below. The Beckman system is described in section 2.2.4.8. (page 80). The Dionex DX500 HPLC system was equipped with a UVD 340U Photodiode Array Detector. Samples were injected onto a C18 column (Waters, 4.6mm x 250mm, particle size: 5µm) using a Dionex AS50 auto-sampler fitted with a 200µl injection loop. Materials eluting from the column were detected using the diode array set at 217nm. Detected peaks were estimated valley to valley and retention times were recorded using the Chromeleon computer program. The solvent system consisted of methanol/water in the ratio of 60:40 run isocratically at a flow rate of 1.0ml/min for 30 minutes. There was an average pressure of 241 bar, which was controlled through a Dionex GP50 gradient pump. Materials eluting from the column were identified through retention time and comparison with known standards.

#### 2.1.4.4. Thin layer chromatography

Normal-phase TLC was performed on 5cm x 10cm silica gel plates (Alugram, 0.20mm, aluminium backed). Aliquots were spotted onto plates, which were developed using a light petroleum: ethyl acetate (30:70) solvent system for a distance



of 7cm. A vanillin/ethanol/sulphuric acid (3g: 160ml: 40ml) solution was used as the staining medium. Staining was visualised after heating with a hot air gun.

### **2.1.5. Refined procedure for azadirachtin-A isolation**

#### *2.1.5.1. Preparation of semi-purified azadirachtin for flash chromatography*

The same method was used to prepare the semi-purified azadirachtin/methanol solution as employed during the preliminary study (see section 2.1.4.1.).

#### *2.1.5.2. Flash column chromatography*

Flash column chromatography was conducted according to the conditions described in the preliminary study (see section 2.1.4.2.). The system was modified in the following respect; after the injection of material onto the column, the initial 1400ml of eluant was collected and discarded. The following 400ml fraction containing the highest purity azadirachtin-A was collected for extraction using dichloromethane (see section 2.1.5.3.). A washing procedure of 600ml methanol was used to purge the column between each run and prevent carryover material.

#### *2.1.5.3. Partitioning with dichloromethane*

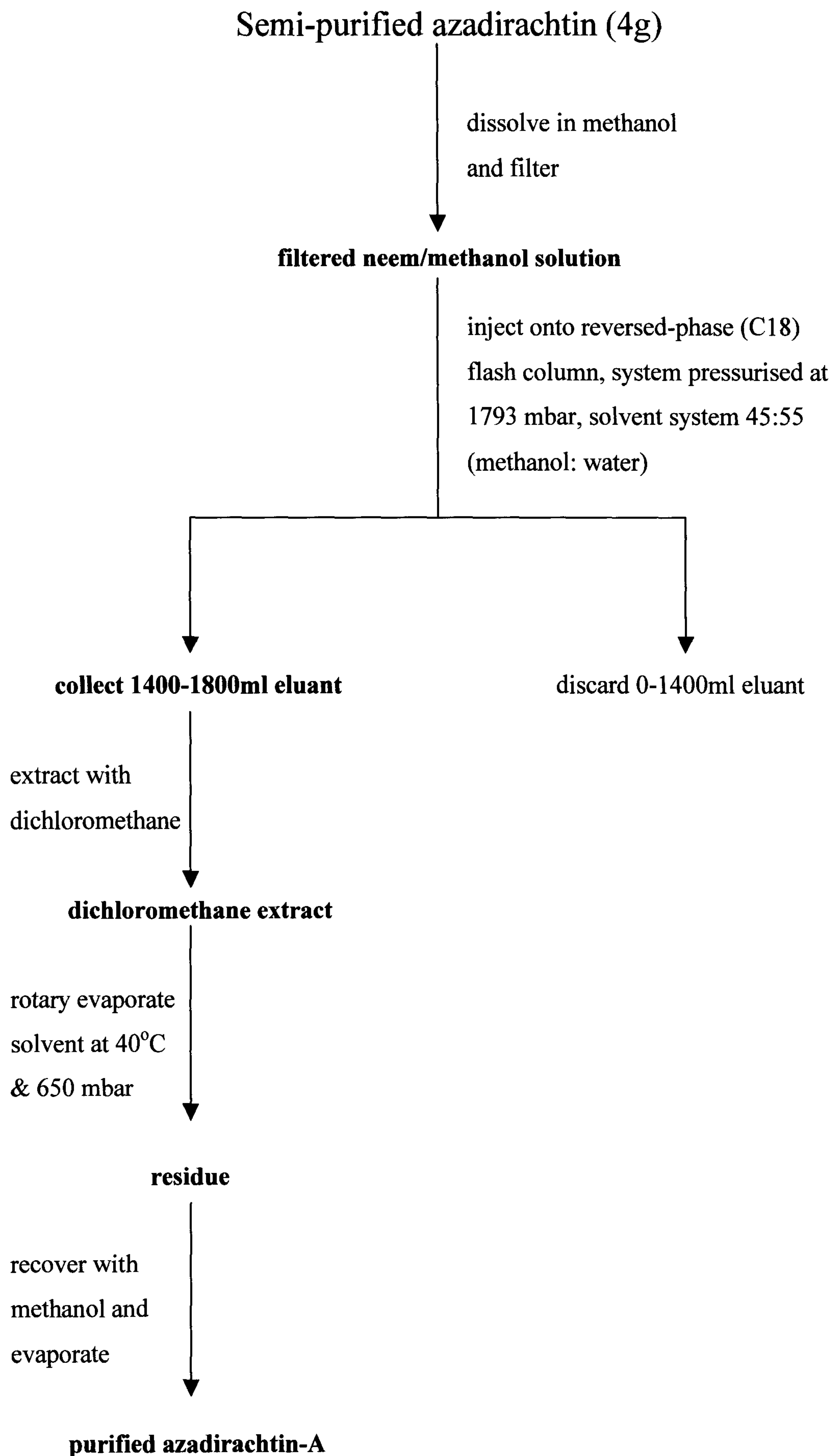
The 400ml fraction was poured into a 2-litre separation funnel. Dichloromethane (200ml) was then added to extract the azadirachtin from the aqueous methanol solution. The mixture was thoroughly mixed and left to stand until 2 distinct layers

had formed, of which the hypophase was decanted and collected. The aqueous hyperphase was partitioned a further two times with the same volume of fresh dichloromethane and the collection procedure repeated. The hypophase collections were pooled and reduced to dryness using a rotary evaporator at 40°C and 650 mbar (Büchi rotavapour R-134) for 20 minutes to yield an off-white viscous residue. This material was recovered into an evaporation basin using 4ml of methanol. The recovery procedure was repeated three times. The methanol solution was left to evaporate to yield approximately 300mg of white micro-crystalline material which was collected and stored at -20°C.

#### 2.1.5.4. Analytical high performance liquid chromatography using the Dionex HPLC system

The material produced from 10 isolation procedures was combined. Samples (10mg) were dissolved in methanol (10ml) and analysed using the Dionex HPLC system (see section 2.1.4.3.) to determine purity and simultaneously, the ultra-violet absorption spectrum.

Figure 2.1.5. **Refined procedure for azadirachtin-A isolation.** Outline of the method developed to isolate azadirachtin-A (~ 95%) from semi-purified azadirachtin, using flash column chromatography.





### **2.1.6. Results**

The material eluting from the flash column was collected into 15, 200ml fractions and assessed using reversed-phase HPLC. Figure 2.1.6.1. shows example chromatograms of the neem limonoids collected within fraction 2 (200-400ml of eluant), fraction 8 (1400-1600ml of eluant) and fraction 14 (2600ml-2800ml of eluant). Azadirachtin-A, identified as having a retention time under analytical HPLC of 5.5 minutes, was shown to be present in all fractions analysed. Initially, azadirachtin-A was shown to elute with limonoids having a higher polarity and therefore, of retention times that were less than 5.5 minutes during analytical HPLC (figure 2.1.6.1.A.). After larger volumes of the mobile phase had been passed through the column, azadirachtin-A was shown to elute at high purity (figure 2.1.6.1.B.) When very high volumes of solvent had been passed through the flash column, azadirachtin-A was shown to elute with limonoids of lower polarity and therefore, of retention times that were greater than 5.5 minutes during analytical HPLC (figure 2.1.6.1.C.).

The limonoids nimbin and salannin were not collected into any of the 15 fractions using the methods described within this chapter. These less polar constituents of semi-purified azadirachtin (identified as being present in figure 2.2.5.2.) were retained on the flash column and required a washing procedure of 100% methanol to be removed.

Figure 2.1.6.2. shows the purity of azadirachtin-A (estimated by peak area during HPLC) as the limonoid was eluted from the flash column into the 15 separate, 200ml fractions. The highest purity of azadirachtin-A was recovered within fractions 8 and 9, which corresponded to a volume of 1400ml-1800ml of solvent being passed through

the column. With the exception of fractions 8 and 9, the purity of azadirachtin-A within each fraction ranged from 3%-83%. Azadirachtin-A was present at purities greater than 50% from fraction 5 to fraction 11 and at purities greater than 80% from fraction 7 to fraction 10. Analysis by TLC (result not presented) revealed that the highest purity of azadirachtin-A also corresponded to the highest quantity of the limonoid being recovered from the column.

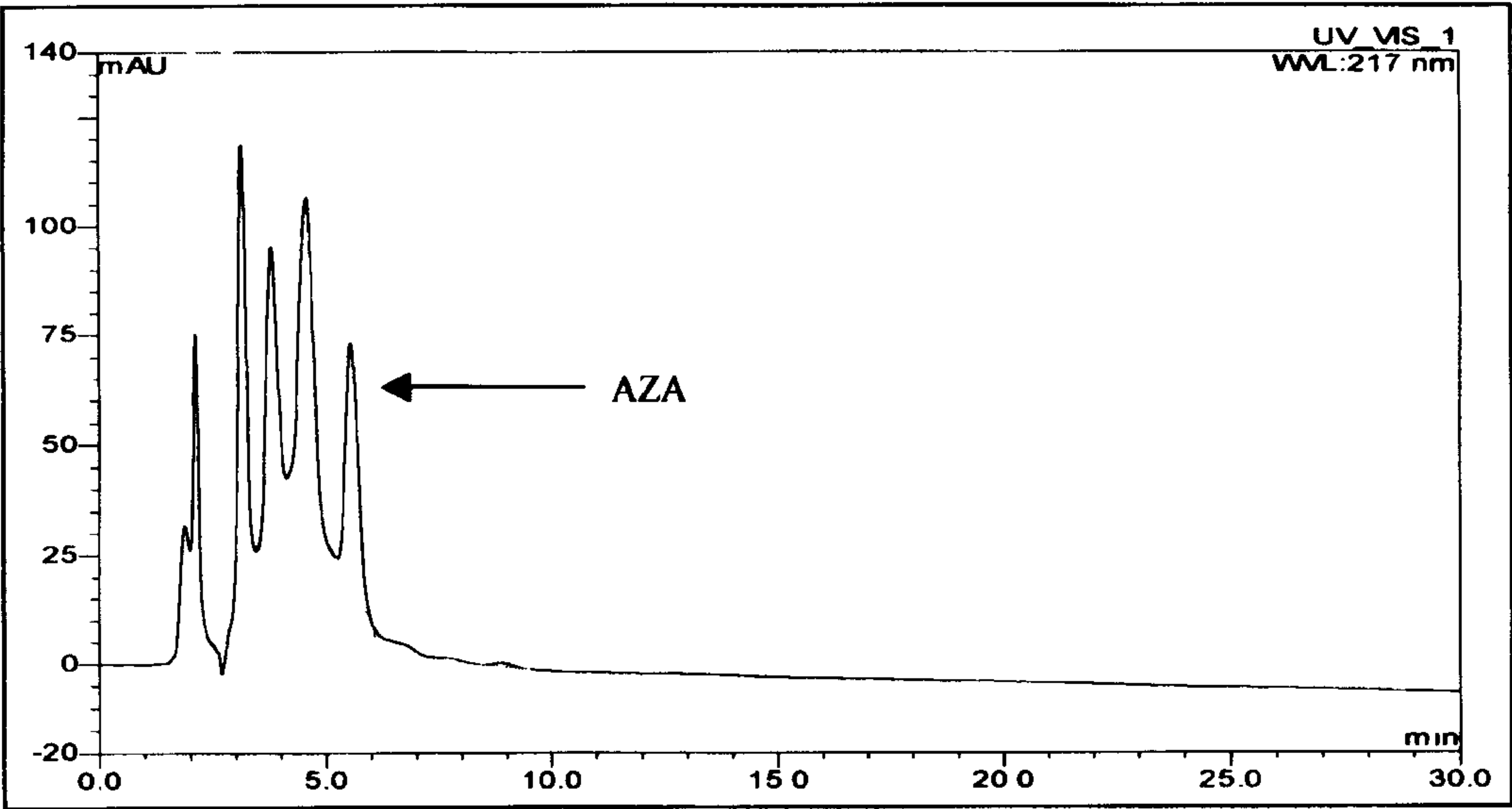
The HPLC chromatogram (figure 2.1.6.3.A.) of purified azadirachtin-A resulting from this procedure is comparable to that of authenticated standards. Purity was determined to be 94.8% as estimated by peak area calculations. The identity and purity of this material was also confirmed by NMR (result not presented). In addition, the purified azadirachtin-A was shown to have an ultra-violet absorption maximum of 216.5nm (figure 2.1.6.3.B.).

The purification process that was developed during this research could achieve a return of 350mg azadirachtin-A (95% pure) from 4g starting material (35% pure) in a process that took approximately 2 hours to complete.

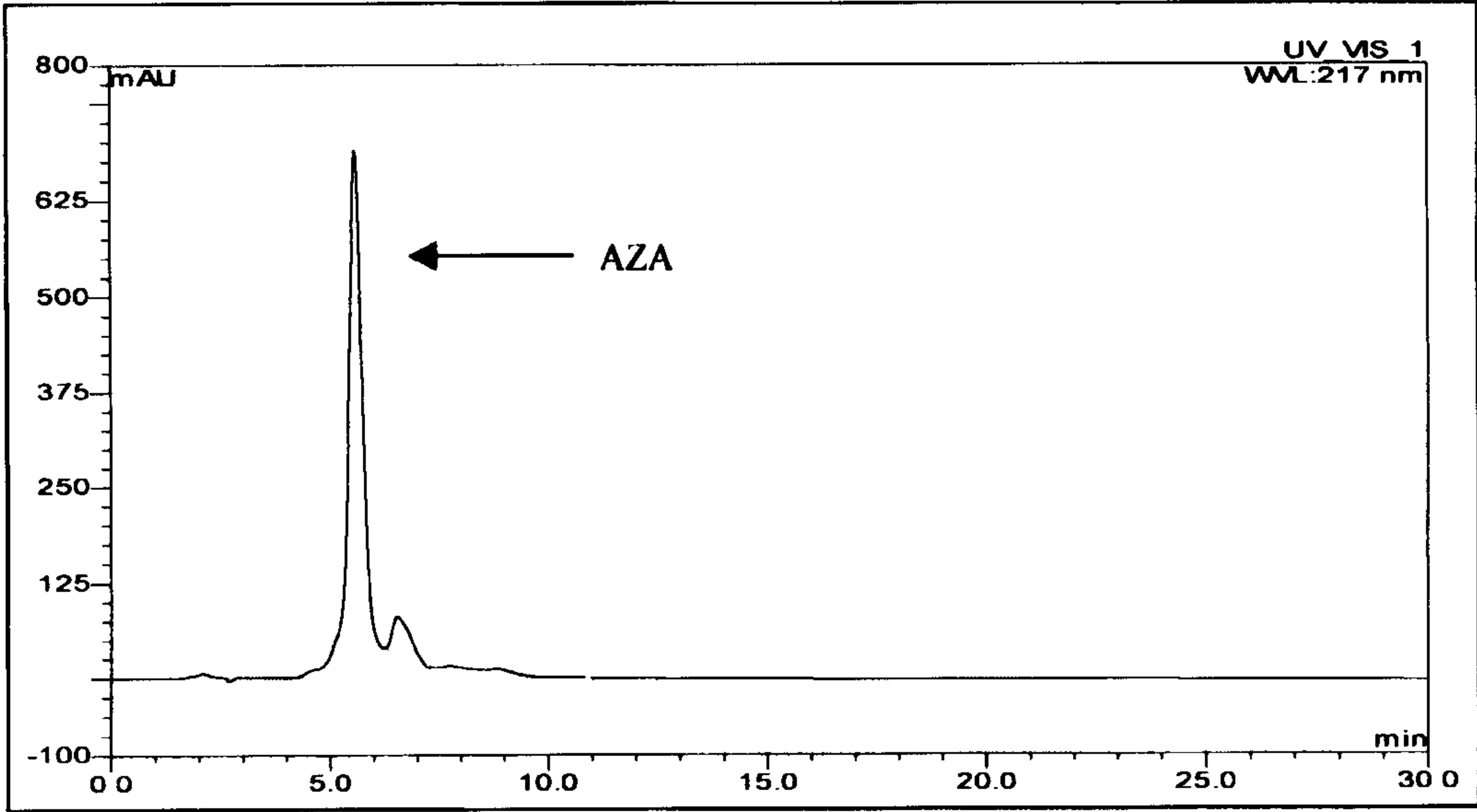
**Figure 2.1.6.1. Analysis of azadirachtin-A and related limonoids eluting from flash column.** Semi-purified azadirachtin was dissolved in methanol and subjected to reversed-phase flash column chromatography (column = C18, solvent system = methanol/water at 45:55) and the resulting eluant collected into 15 separate 200ml fractions. Aliquots of these fractions were directly assessed using the Dionex HPLC system as described in section 2.1.4.3. (column = C18, solvent system = methanol/water at 60:40). The purity of azadirachtin was quantified by peak area (valley to valley) assessments. **(A)** Azadirachtin-A and related limonoids within fraction 2 (200-400ml of eluant). **(B)** Azadirachtin-A and related limonoids within fraction 8 (1400-1600ml of eluant). **(C)** Azadirachtin-A and related limonoids within fraction 14 (2600-2800ml of eluant).

**Key:** AZA = Azadirachtin-A (retention time = 5.5 minutes)

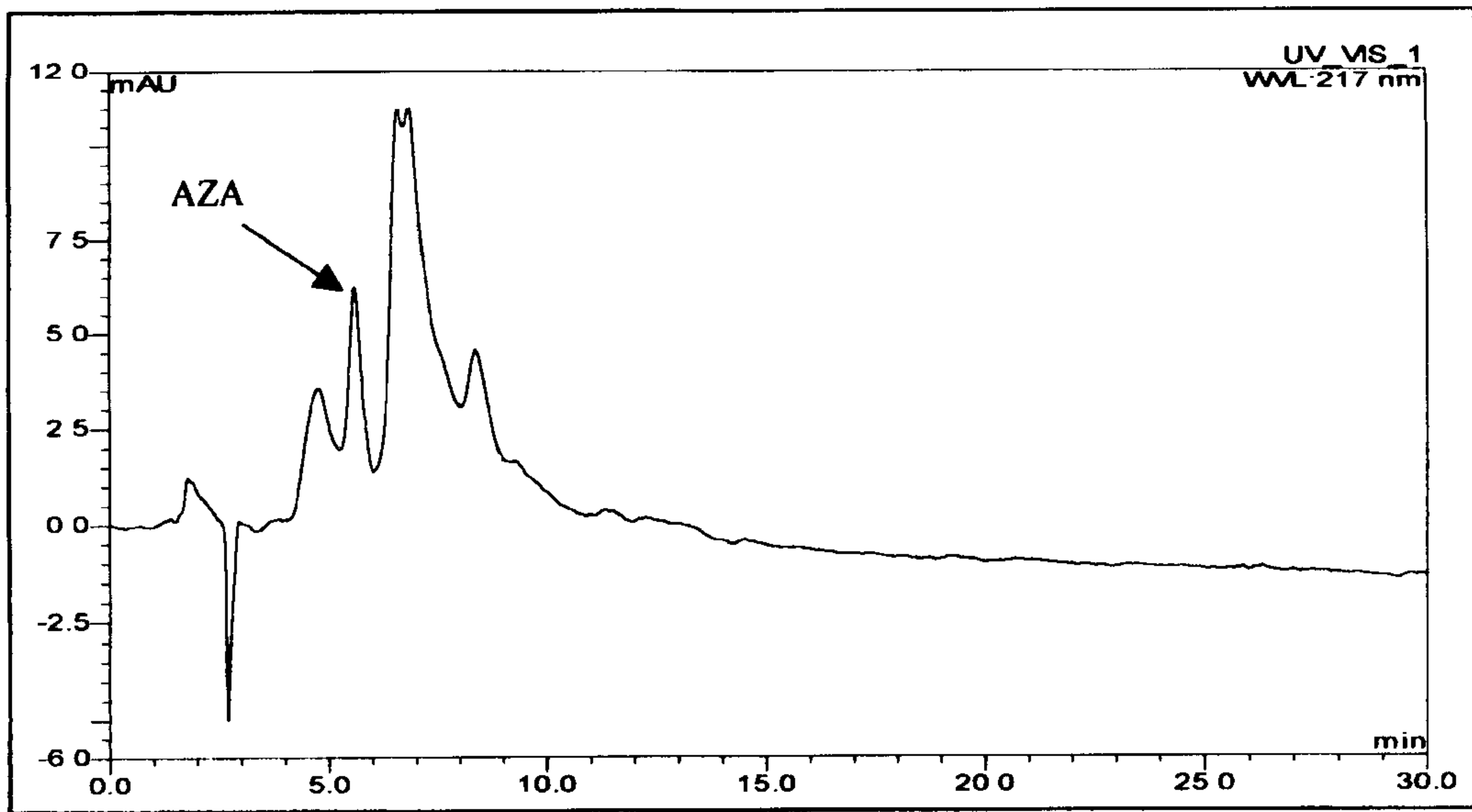
(A)



(B)

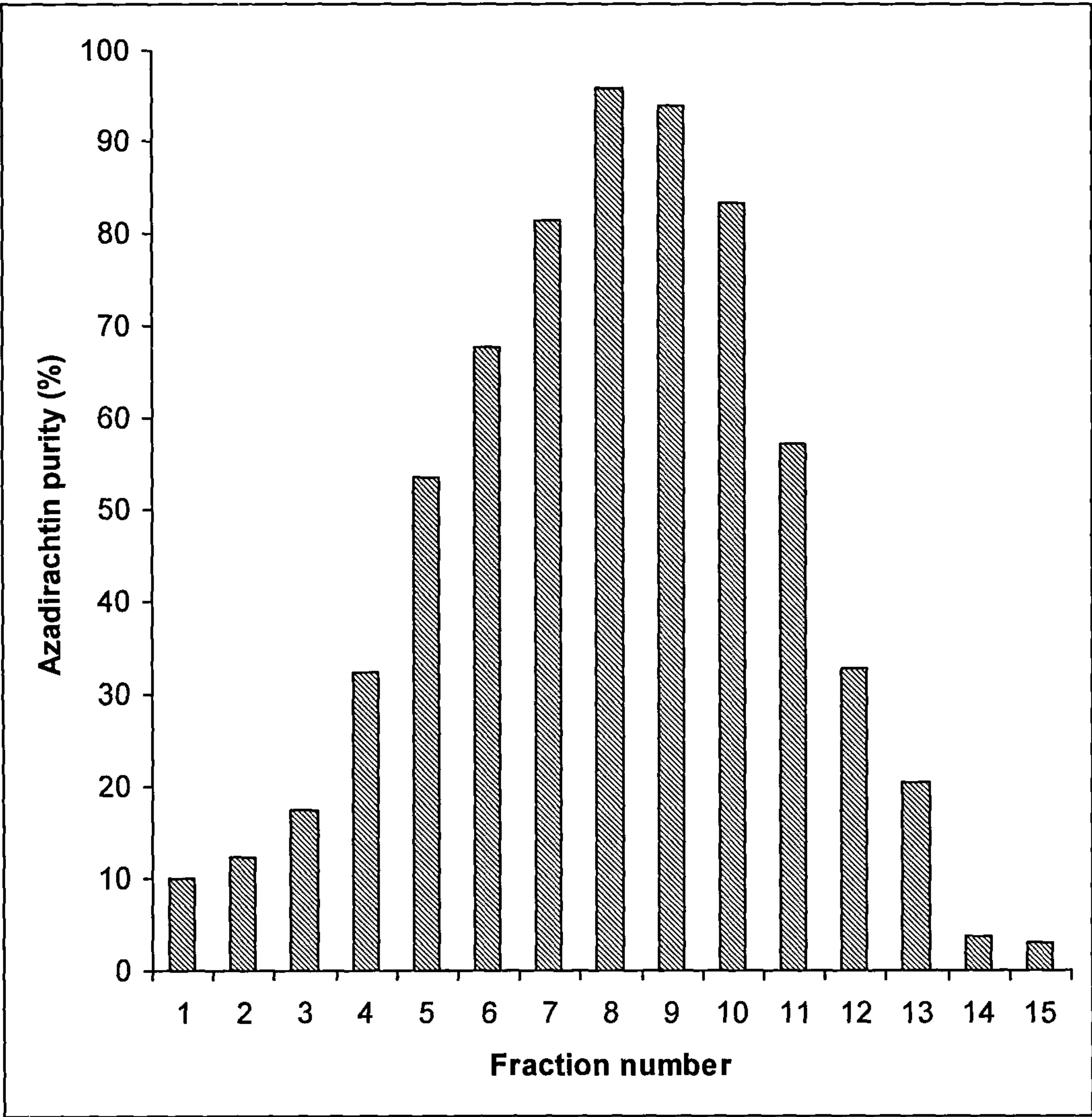


(C)





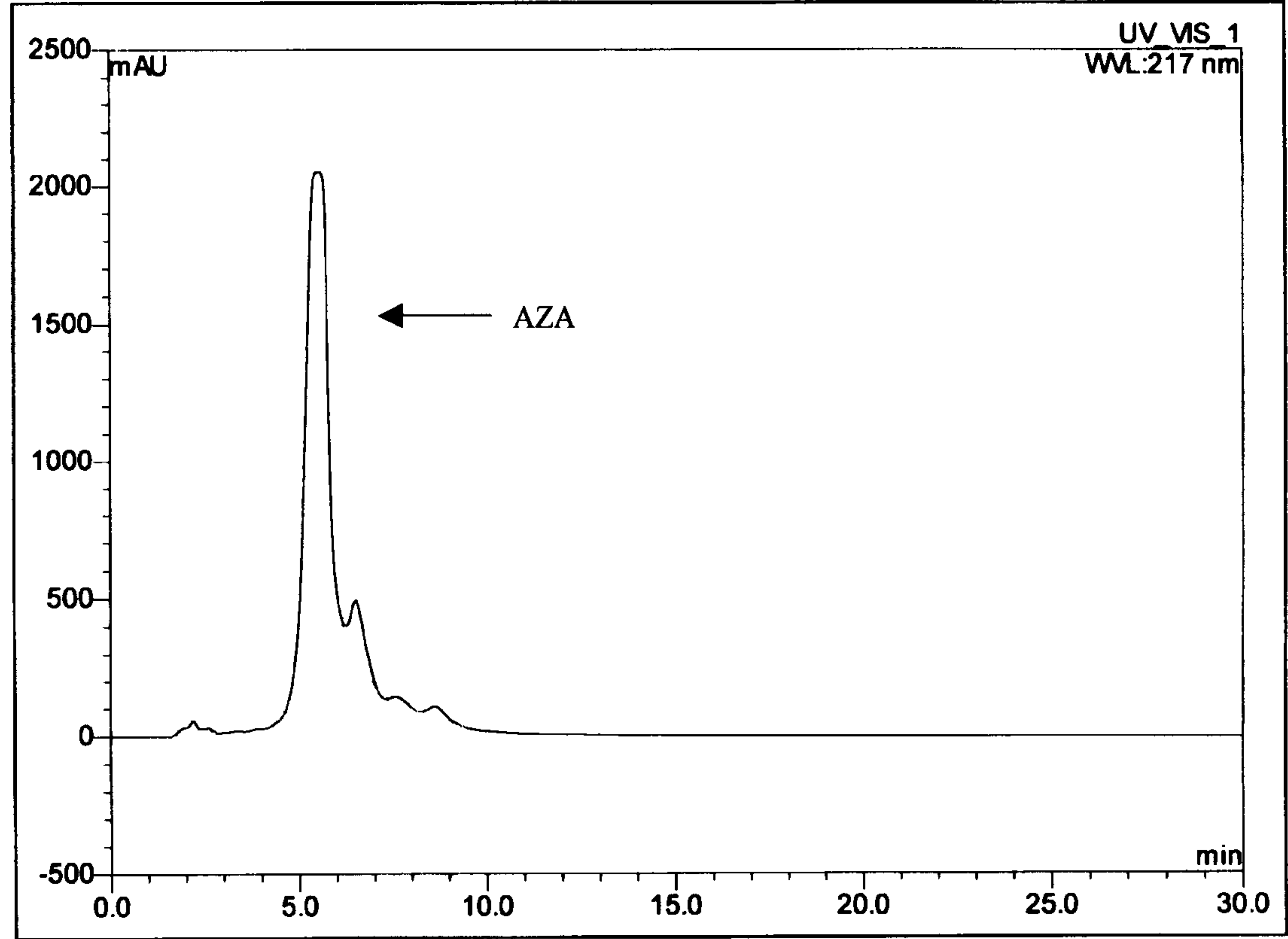
**Figure 2.1.6.2. Purity of azadirachtin-A eluting from flash column.** Semi-purified azadirachtin was dissolved in methanol and subjected to reversed-phase flash column chromatography (column = C18, solvent system = methanol/water at 45:55) and the resulting eluant collected into 15 separate 200ml fractions. Aliquots of these fractions were directly assessed using the Dionex HPLC system as described in section 2.1.4.3. (column = C18, solvent system = methanol/water at 60:40). The purity of azadirachtin was quantified by peak area (valley to valley) assessments. Increasing fraction number represents increasing volume eluted through the column in 200ml increments.



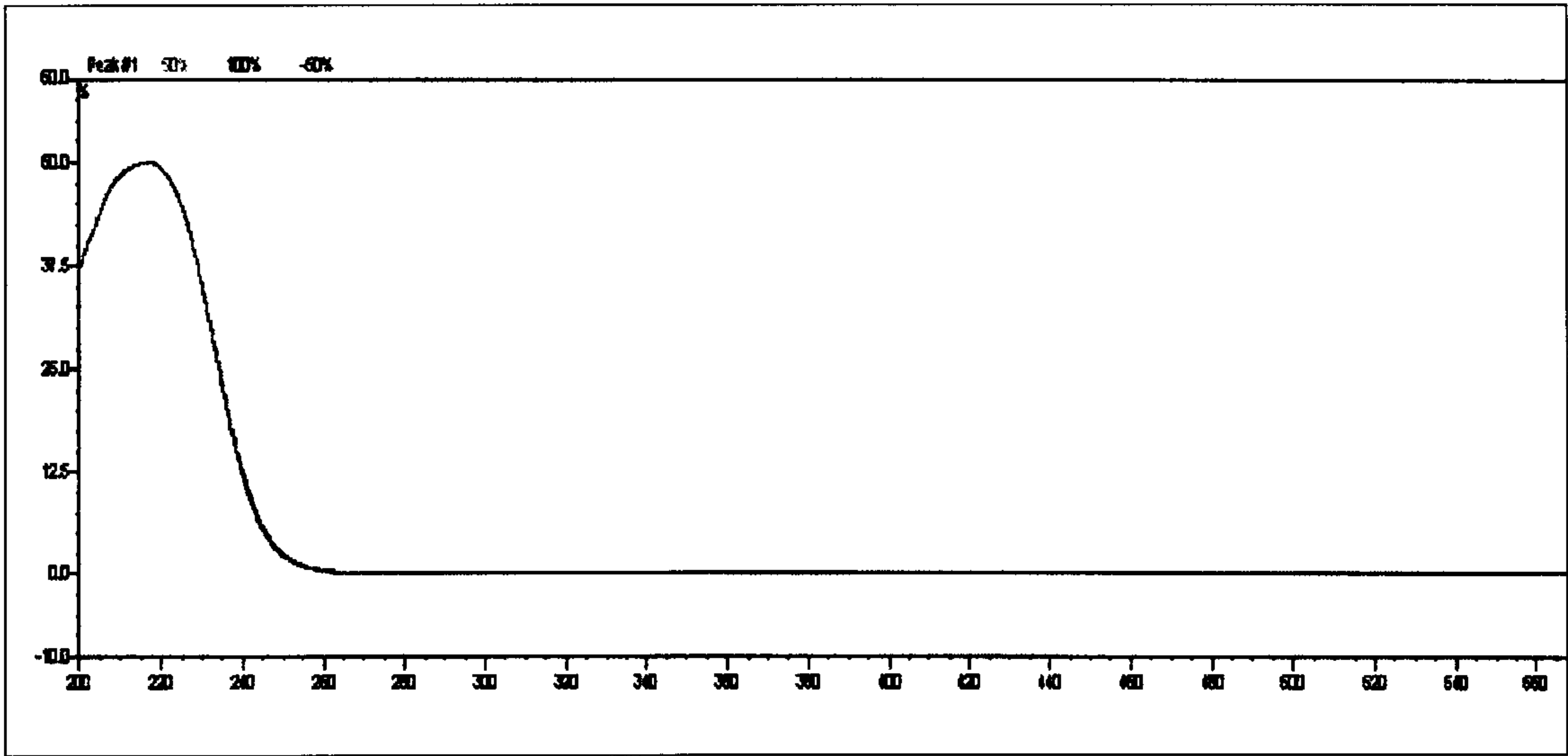
**Figure 2.1.6.3. Purified azadirachtin-A resulting from flash column chromatography.** Semi-purified azadirachtin was dissolved in methanol and subjected to reversed-phase flash column chromatography (column = C18, solvent system = methanol/water at 45:55). The material eluting from the column after 1400ml-1800ml was collected and repeatedly partitioned between dichloromethane. The dichloromethane was evaporated-off to leave azadirachtin-A. The purification process was repeated using fresh semi-purified azadirachtin until 2.5g of pure azadirachtin-A had been obtained. The azadirachtin-A obtained from all purification procedures was combined. Samples were dissolved in methanol and aliquots assessed using the Dionex HPLC system as described in section 2.1.4.3. (column = C18, solvent system = methanol/water at 60:40). The purity of azadirachtin-A was quantified by peak area (valley to valley) assessments. **(A)** Chromatogram of purified azadirachtin-A, purity = 94.8%. **(B)** The ultra-violet absorption spectrum of purified azadirachtin-A,  $\lambda_{\text{max}} = 216.5\text{nm}$

Key: AZA = Azadirachtin-A (retention time = 5.5 minutes)

(A)



(B)





### **2.1.7. Discussion**

As previously indicated, the isolation of azadirachtin from the seeds of *Azadirachta indica* has always been regarded as difficult. The most common method, which relies on semi-preparative and preparative isolation by HPLC is expensive, time-consuming and of low-yield (Hein & Hummel, 2000 and Thejavathi *et al.*, 1995). Therefore, alternatives have been investigated, however these often suffer from the same disadvantages (Ambrosino *et al.*, 1999; Jarvis *et al.*, 1999 and Lou *et al.*, 1999).

The results presented within this section show that purification based on flash chromatography (outlined in figure 2.1.5.) is an approach that is suitable for isolating pure azadirachtin-A. The principle of the method relies on separating the various neem constituents according to their polarity, in particular their oxygen functionality.

The method that was developed was capable of rapidly isolating large quantities of 95% pure azadirachtin-A from a 35% pure source. In addition, it was observed that small amounts of 97% pure material could also be recovered. In terms of the maximum purity that can be obtained, isolation using flash chromatography can be regarded as inferior to those based on HPLC, which can achieve purities of 99% (Nisbet, 1991). However, the method developed by this work does provide considerable benefits regarding the speed, simplicity and yield of the purification procedure.

## **2.2. Quantitative estimation of neem seed extracts**

### **2.2.1. Introduction**

Only a few methods can be used to quantify azadirachtin and its related limonoids within neem extracts. Typically, these are based on reversed phase high performance liquid chromatography, although supercritical fluid chromatography and electrospray mass spectrometry have also been employed (Ambrosino *et al.*, 1999 and Warthen *et al.*, 1984). Characteristically, the quantification of azadirachtin using these methods suffer from disadvantages of being slow and non-specific. This is because azadirachtin absorbs at low ultra-violet wavelengths (i.e. <220nm, see figure 2.1.6.3.B.). Therefore, interference from chromophoric groups of other molecules present within extracts can be high. As a consequence, to accurately assess the limonoid within various mixtures, chromatographic separation is required, which is time consuming.

These problems have stimulated the development of alternative approaches that can be used rapidly and accurately to quantify the limonoid. Notably, Dai *et al.*, (1999) has developed an assay that involves reacting azadirachtin with acidified vanillin solutions to produce coloured complexes. The absorbance of these solutions, at a particular wavelength, corresponding directly to azadirachtin content. Therefore, it has been suggested that this method offers the possibility of a procedure that is both rapid and specific for azadirachtin estimation. A considerable advantage of the methodology being that it may be able to quantify the limonoid within many samples simultaneously.

### **2.2.2. Aims**

The second aim of chapter 2 was to quantify azadirachtin and related limonoids (nimbin and salannin) using the staining procedure developed by Dai *et al.*, (1999) and to compare the derived estimations with quantification based on HPLC. In addition, both methods would be used to examine three crude neem seed extracts (semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil), which contained different amounts of azadirachtin. This research would indicate the suitability and reliability of the technique in rapidly quantifying azadirachtin within crude neem seed extracts and during environmental-fate studies conducted at later points in the project.

### **2.2.3. Materials**

#### **2.2.3.1. General materials**

Vanillin (4-hydroxy-3-methoxybenz-aldehyde) was purchased from Sigma-Aldrich (Poole, Dorset, UK). Semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil was purchased from ShriDisha Biotech, Hyderabad, India. All neem extracts were used without further modification. Nimbin and salannin were supplied by Dr. Robin Strang (University of Glasgow). Azadirachtin-A was obtained from flash column chromatography, described in section 2.1. All standards used had a purity of approximately 95% as estimated by HPLC.

## **2.2.4. Experimental**

### **2.2.4.1. Extraction of azadirachtin and related limonoids from crude neem seed extracts**

Semi-purified azadirachtin, de-fatted neem seed kernel extract or neem seed oil (1g) in replicates of three were added to methanol (5ml). The limonoids were extracted into the methanol using an Ultra Turrax homogeniser (6000rpm for 20 seconds) or by vortex mixing (20 seconds) where appropriate. The resulting extracts of crude neem materials were centrifuged (3000rpm for 3 minutes) to separate the particulate material and oil from the methanol. This methanol phase was collected and the extraction procedure was repeated on the partially extracted material a further 2 times. Extracts were pooled and filtered using a syringe fitted with a 0.2µm syringe filter.

### **2.2.4.2. Preparation of methanol extracts for the vanillin assay**

An aliquot of each neem/methanol extract (see section 2.2.4.1.) was appropriately diluted with methanol. A suitable volume of this dilution was added to distilled water (1ml) and the limonoids within partitioned into dichloromethane (1ml) by vortex mixing for 20 seconds. The mixture was spun by centrifuge (3000rpm for 1 minute) into separate phases. The hypophase was collected by pipette and the limonoids remaining within the aqueous hyperphase were partitioned into fresh dichloromethane a further two times using this method. The hypophase collections were pooled and diluted with an appropriate volume of dichloromethane for analysis.



#### 2.2.4.3. Preliminary vanillin assay

To a dichloromethane solution (0.7ml) containing pure neem standard (azadirachtin, nimbin or salannin) or crude neem extract (extracted from semi-purified azadirachtin, de-fatted neem seed kernel extract or neem seed oil (see sections 2.2.4.1. and 2.2.4.2.)), a methanol solution (0.2ml) containing vanillin (0.02g/ml) was added. After shaking manually for 5 seconds, the mixture was left at room temperature for 2 minutes. Concentrated sulphuric acid (20M) was then added in 3 separate additions (0.1ml each) to produce a coloured solution. The mixture was manually shaken for 10 seconds following each addition. The resulting two-layer mixture was converted into a homogenous solution through the addition of methanol (0.7ml). The solutions were allowed to stand at room temperature for 5 minutes to allow the colour to stabilise. After this period, the absorbance was scanned over the wavelength range of 400nm to 700nm, using a Shimadzu, UV-1201, UV-VIS spectrophotometer equipped with a 10mm glass cell and the  $\lambda_{\text{max}}$  was measured.

#### 2.2.4.4. Calibration curve of azadirachtin subjected to vanillin assay

A calibration curve was generated from standard azadirachtin-A solutions in dichloromethane (concentration range = 0.01mg/ml-0.5mg/ml) that were subjected to the vanillin staining procedure described below in section 2.2.4.5.

#### 2.2.4.5. Finalised vanillin assay

The vanillin quantification assay was conducted according to the conditions described in the preliminary study (see section 2.2.4.3.). The system was modified in the following respect; after the colorized solution was stabilised for 5 minutes, absorption was measured at 574nm using a Cecil (CE 272) linear readout, ultraviolet spectrophotometer equipped with a tungsten lamp. To obtain a blank solution, the test solution was substituted with an equal volume of dichloromethane in the above procedure. Quantification was achieved by correlating absorbance against azadirachtin concentration plotted on a calibration curve (figure 2.2.5.4.).

#### 2.2.4.6. The vanillin assay using methanol

The vanillin quantification assay was conducted according to the conditions described in section 2.2.4.3. The system was modified by replacing dichloromethane with methanol throughout the assay.

#### 2.2.4.7. Calculation of extinction coefficients

The vanillin assay was conducted using the same methods as described in section 2.2.4.5. with the following modification. The level of absorption was measured at the  $\lambda_{\text{max}}$  for each limonoid (azadirachtin-A = 574nm, nimbin = 437nm and salannin = 574nm). The extinction coefficient was then calculated using the equation:

$$A = \epsilon \times c \times l$$

Where  $A$  is absorbance (AU),  $\epsilon$  is the molar extinction coefficient ( $\text{l.mole}^{-1}$ ),  $c$  is the molar concentration of the solution ( $\text{mole.l}^{-1}$ ) and  $l$  is the pathlength (cm). The precise extinction coefficient was determined by taking the mean value of four replicates.

#### 2.2.4.8. Analytical high performance liquid chromatography using the Beckman HPLC system

Analytical reversed-phase HPLC was conducted using one of two systems. The Beckman system is described below. The Dionex system is described in section 2.1.4.3. (page 61). Samples were injected onto a C18 column (Waters, 4.6 x 250mm, particle size: 5 $\mu\text{m}$ ) using a Beckman (210A) injector fitted with a 200 $\mu\text{l}$  injection loop. Materials eluting from the column were detected using a Beckman (166), programmable detector, set at 217nm that was equipped with a Beckman (406), analogue interface module. Detected peaks were estimated valley to valley and retention times were recorded using a NEC PC-8300 module linked to a Servogor chart recorder. The solvent system consisted of methanol/water run in a gradient elution. The ratio of methanol to water was increased from 60% to 100% over a 10-minute period and returned from 100% to 60% over a 2-minute period. There was a flow rate of 1ml/min, at an average pressure of 207 bar. Pressure was controlled through a Beckman (110B) solvent delivery module. Materials eluting from the column were identified through retention time and comparison with known standards.

## **2.2.5. Results**

### **2.2.5.1. Analysis of pure limonoids and crude neem seed extracts by high performance liquid chromatography**

Figure 2.2.5.1. shows that the pure neem standards azadirachtin, nimbin and salannin were efficiently separated by the HPLC conditions described in the methods. Each limonoid was shown to produce a single symmetrical peak of absorbance. Azadirachtin had a retention time of 12 minutes, nimbin = 15 minutes and salannin = 17 minutes. Based on HPLC analysis, each standard was shown to contain only negligible amounts of contaminating materials.

Crude neem seed extracts were extracted into methanol and analysed by HPLC (figures 2.2.5.2.). The extracts were predominately composed of azadirachtin-A, nimbin and salannin. However, numerous unidentified limonoids of variable polarity were also shown to be present. In particular, de-fatted neem seed kernel extract contained a large quantity of very polar materials that eluted from the HPLC column shortly after sample injection.

Figure 2.2.5.9. gives details of the quantities of azadirachtin, nimbin and salannin, as estimated by peak area, within the crude neem seed extracts. Semi-purified azadirachtin contained the greatest concentration of azadirachtin-A (156mg/g). The concentration of this limonoid within de-fatted neem seed kernel extract and neem seed oil was 0.87mg/g and 0.39mg/g respectively. Therefore, in terms of percentage purity, azadirachtin-A was present within crude neem seed extracts at levels ranging



from 0.04%-16% (w/w). There were low concentrations of nimbin present within the various crude neem seed extracts. Semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil contained 0.07mg/g, 0.12mg/g and 0.06mg/g respectively. Therefore, in terms of percentage, all extracts contained approximately 0.01% (w/w) or less of this limonoid. The amounts of salannin within the extracts ranged from 0.01%-0.05% (w/w). Semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil contained 0.13mg/g, 0.46mg/g and 0.43mg/g of this limonoid respectively. When the combined concentrations of azadirachtin, nimbin and salannin within each extract were calculated, semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil contained 156.5mg/g, 1.45mg/g and 0.88mg/g of the limonoids respectively.

#### 2.2.5.2. Analysis of pure limonoids and crude neem seed extracts by vanillin assay

When azadirachtin-A, nimbin and salannin were subjected to the vanillin assay, all the pure standards formed coloured complexes. The colour of the solutions produced after the first five minutes of the reaction were, azadirachtin-A = purple/blue, nimbin = grey/blue and salannin = grey/blue. The reference methanol solutions that contained no neem materials also reacted to produce a very pale blue coloured solution. All solutions gradually turned purple over time. As the reaction continued with time, the solutions became dark purple and almost opaque in appearance.

Figure 2.2.5.3. shows absorbance, in the visible range, of the major neem limonoids following reaction with vanillin. The spectra illustrate that after 5 minutes, both azadirachtin and salannin produced a single absorption maximum at 574nm, although

the peak in salannin's spectrum was considerably broader than azadirachtin's. Nimbin was shown to respond to the vanillin assay to produce absorption maxima at 437nm and 617nm, although the level of absorbance of the latter peak was less than that of the former. Blank methanol solutions produced a single absorption maximum at 569nm.

When the pure limonoids were subjected to the assay and the absorbance measured at the  $\lambda_{\text{max}}$  for each standard, azadirachtin-A, nimbin and salannin had molar extinction coefficients of  $1.22 \times 10^3 \text{ l.mole}^{-1}$ ,  $2.89 \times 10^3 \text{ l.mole}^{-1}$  and  $2.5 \times 10^3 \text{ l.mole}^{-1}$  respectively (figure 2.2.5.4.C).

Under the conditions of the assay, a good linearity was found between absorbance at 574nm and the concentration of azadirachtin within dichloromethane solutions in the ranges of 0.01mg/ml-0.5mg/ml.

The stability of the coloured solution in terms of the  $\lambda_{\text{max}}$  and the corresponding level of absorbance were assessed over a 35-minute period. Figures 2.2.5.4.A. shows that the  $\lambda_{\text{max}}$  for azadirachtin and the blank methanol solution was stable during this period, remaining at approximately 574nm and 569nm respectively. The  $\lambda_{\text{max}}$  for salannin was shown not to be stable. Initially, salannin responded to the assay to produce an absorption maximum at 592nm. However, as the reaction of the assay progressed, the wavelength of this maximum was shown to decline towards 557nm. Nimbin produced a different response to the assay than azadirachtin-A and salannin. Initially, this limonoid reacted with vanillin to create two relatively stable absorption maxima, one at 437nm and a second at 615nm. However, after 10 minutes, a third

stable absorption maximum at 519nm was formed. After 15 minutes and for the remainder of the trial, the initial two absorption maxima dissipated to leave the single peak in absorption at approximately 516nm.

Figure 2.2.5.4.B. shows the change in the level of absorbance of the  $\lambda_{\max}$  over time. Generally, it can be seen that the absorbance of the  $\lambda_{\max}$  was not stable. The absorption maximum of the blank reference solution increased slightly from 0.198AU-0.216AU throughout the 35-minute observation period. Similarly, the  $\lambda_{\max}$  of azadirachtin increased from 0.637AU-0.674AU as the reaction of the assay progressed. In contrast, the absorption maximum of salannin decreased from an initial 0.517AU to 0.489AU at 15 minutes. After this point, the absorption maximum increased to 0.502AU. The primary and secondary absorption maxima of nimbin decreased, from 1.376AU-1.063AU and 0.931AU-0.844AU respectively, until they were dissipated at 15 minutes. Nimbin's tertiary absorbance maximum increased from 0.790AU at 10 minutes (when it was first recorded) to 0.875AU at 35 minutes.

The crude neem seed extracts also reacted with the vanillin assay to produce coloured solutions (figure 2.2.5.5.). Analysis of absorption bands revealed that semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil had spectra that centred at 578nm, 563nm and 558nm respectively. The absorbance spectrum of semi-purified azadirachtin was shown to be similar in shape to that of the purified azadirachtin-A standard. This would indicate that this crude neem seed extract is largely composed of this limonoid. The absorbance spectrum of de-fatted neem seed kernel extract and neem seed oil both showed considerable band broadening on both

sides of the  $\lambda_{\text{max}}$  of azadirachtin-A (574nm). This would indicate the presence of other limonoids.

If dichloromethane is replaced with methanol during the vanillin assay, the absorption spectra of the crude neem seed extracts is changed. As shown by figure 2.2.5.6., the spectra formed using this modified procedure lack any distinctive peak in absorption. Nevertheless, the crude neem seed extracts reacted with vanillin to produce minor maxima at 503nm and 580nm (semi-purified azadirachtin), 495nm (de-fatted neem seed kernel extract) and 512nm (neem seed oil).

As all of the neem limonoids tested reacted with the vanillin assay to produce coloured complexes, the concentration of azadirachtin and related limonoids within the different neem seed extracts was estimated (figure 2.2.5.8.). The assay estimated that semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil contained 912mg/g, 184mg/g and 34mg/g respectively.



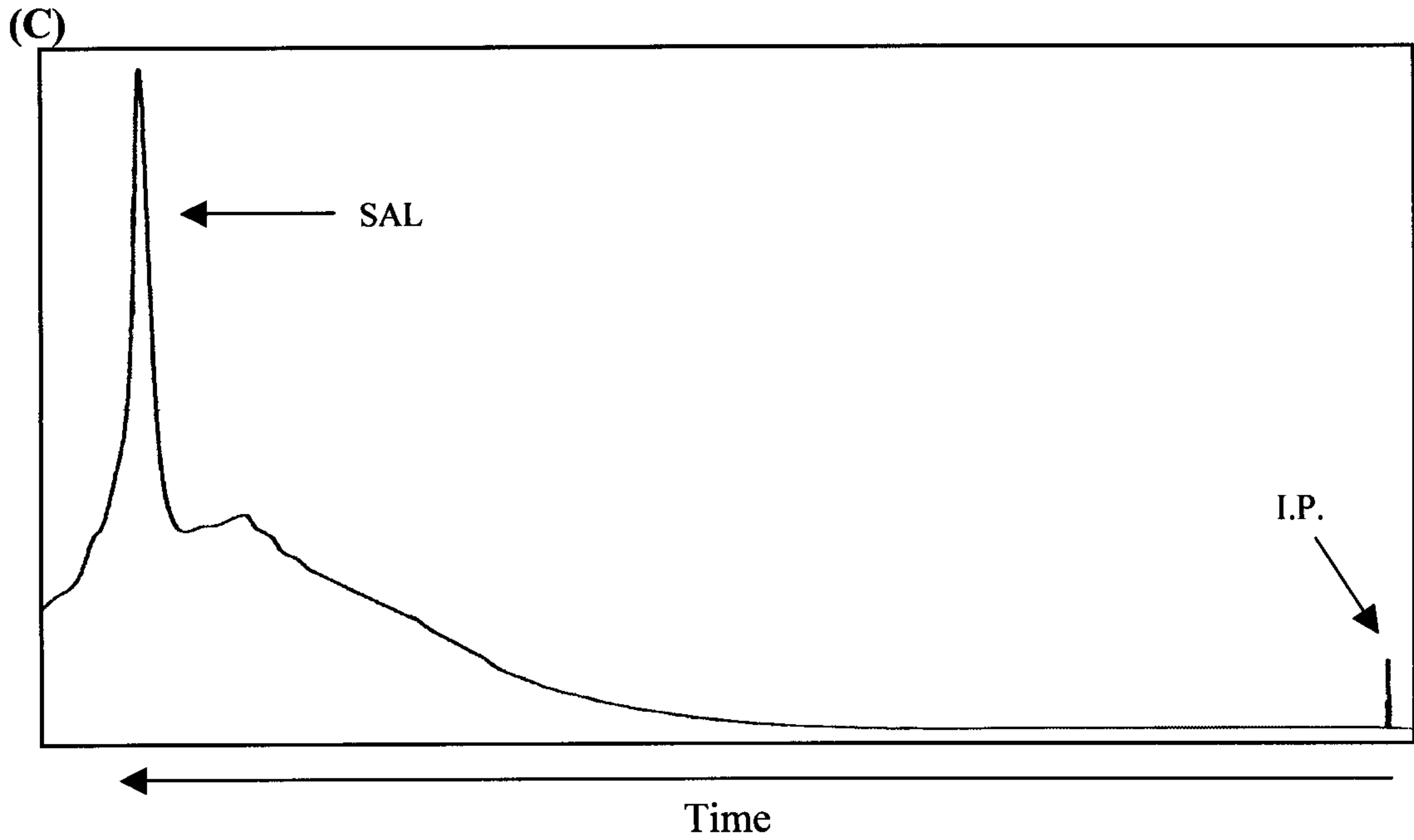
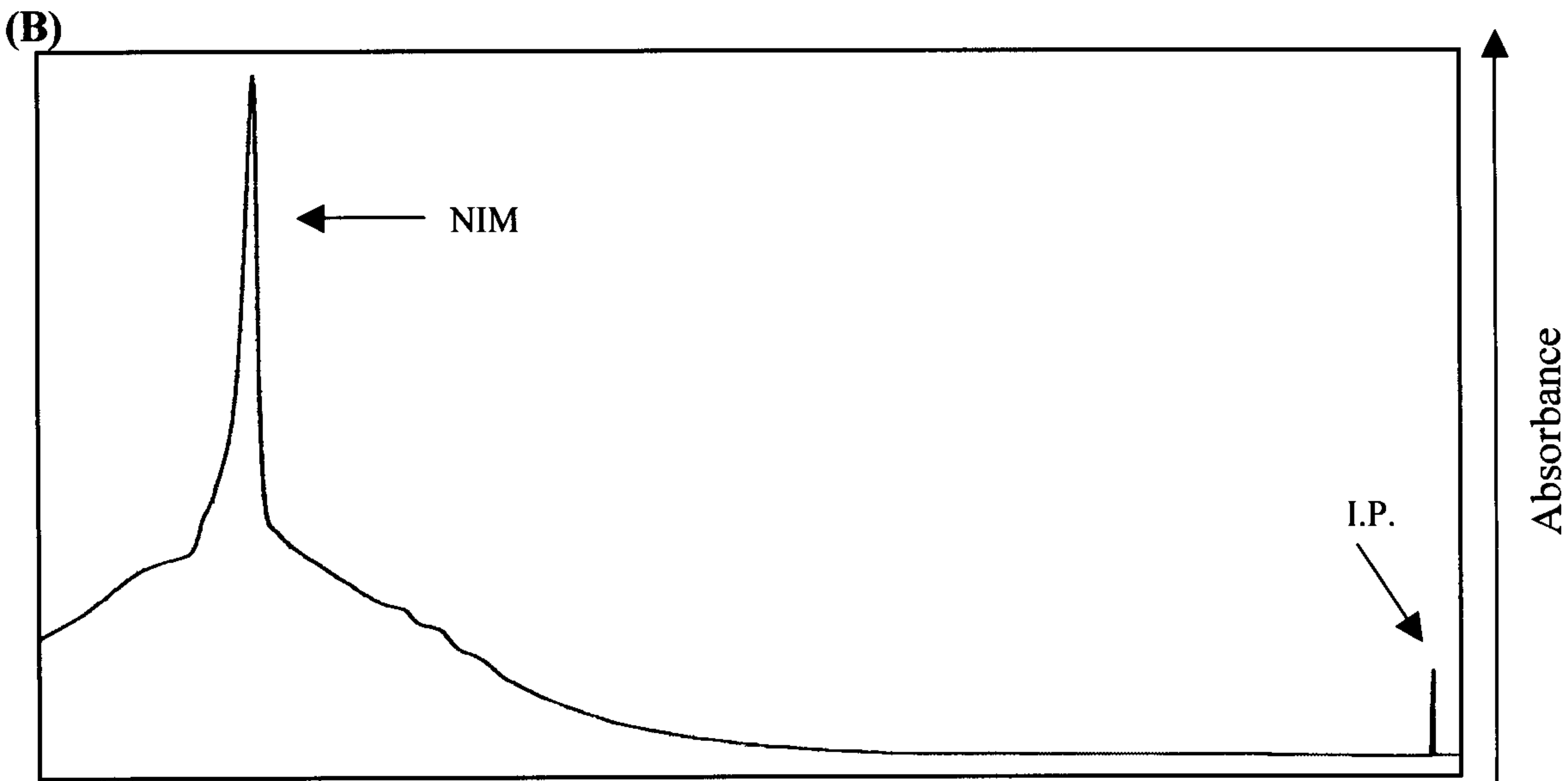
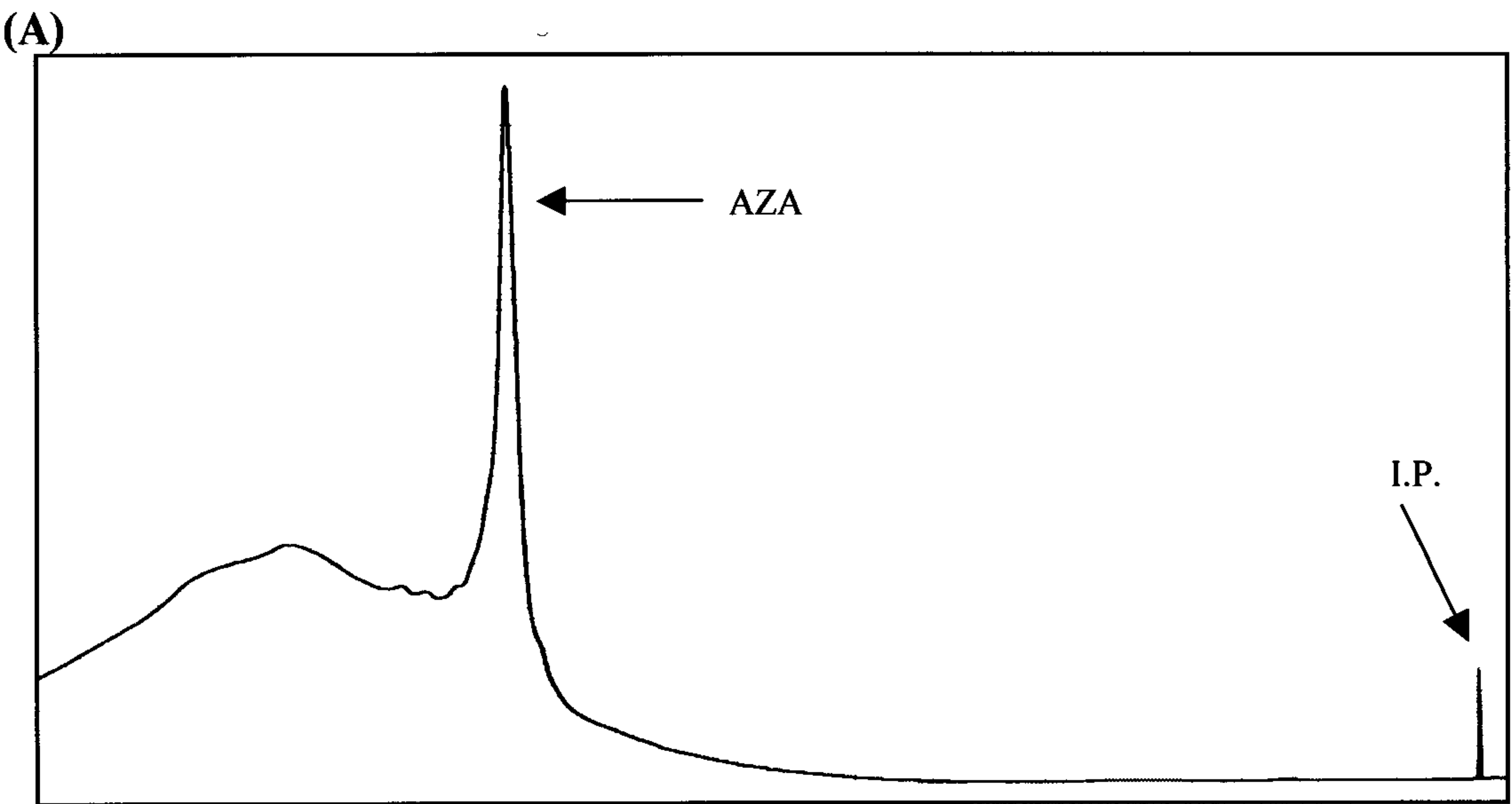
Figure 2.2.5.1. **HPLC chromatograms of the major limonoids of neem.** Azadirachtin-A, nimbin and salannin were dissolved in volumes of methanol. Aliquots were assessed using the Beckman HPLC system as described in section 2.2.4.8. (column = C18, solvent system = methanol/water. The percentage of methanol within the mobile phase was increased from 60% to 100% over a 10-minute period and returned from 100% to 60% over a 2-minute period). **(A)** Azadirachtin-A, retention time: 11.9 minutes. **(B)** Nimbin, retention time: 15.0 minutes. **(C)** Salannin, retention time: 16.6 minutes. The increasing baseline absorption corresponds to the increasing concentration of methanol within the solvent system.

Key: I.P. = Injection point

AZA = Azadirachtin-A

NIM = Nimbin

SAL = Salannin



**Figure 2.2.5.2. HPLC chromatograms of crude neem seed extracts.** The limonoids within semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil were extracted into methanol. Aliquots were assessed using the Beckman HPLC system as described in section 2.2.4.8. (column = C18, solvent system = methanol/water. The percentage of methanol within the mobile phase was increased from 60% to 100% over a 10-minute period and returned from 100% to 60% over a 2-minute period). **(A)** Semi-purified azadirachtin. **(B)** De-fatted neem seed kernel extract. **(C)** Neem seed oil. The increasing baseline absorption corresponds to the increasing concentration of methanol within the solvent system.

Key: I.P. = Injection point

AZA = Azadirachtin-A (Retention time: 11.9 minutes)

NIM = Nimbin (Retention time: 15.0 minutes)

SAL = Salannin (Retention time: 16.6 minutes)

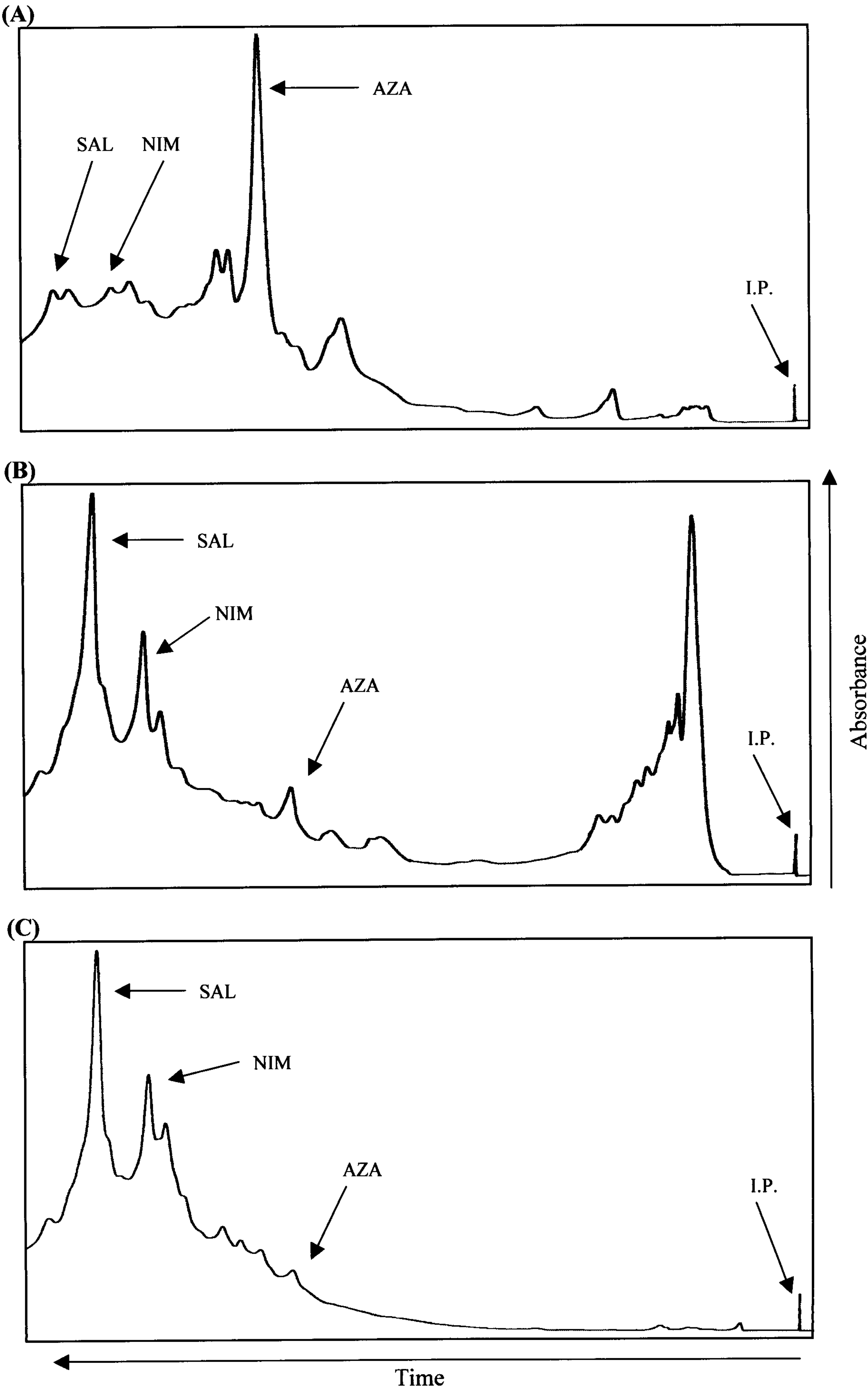
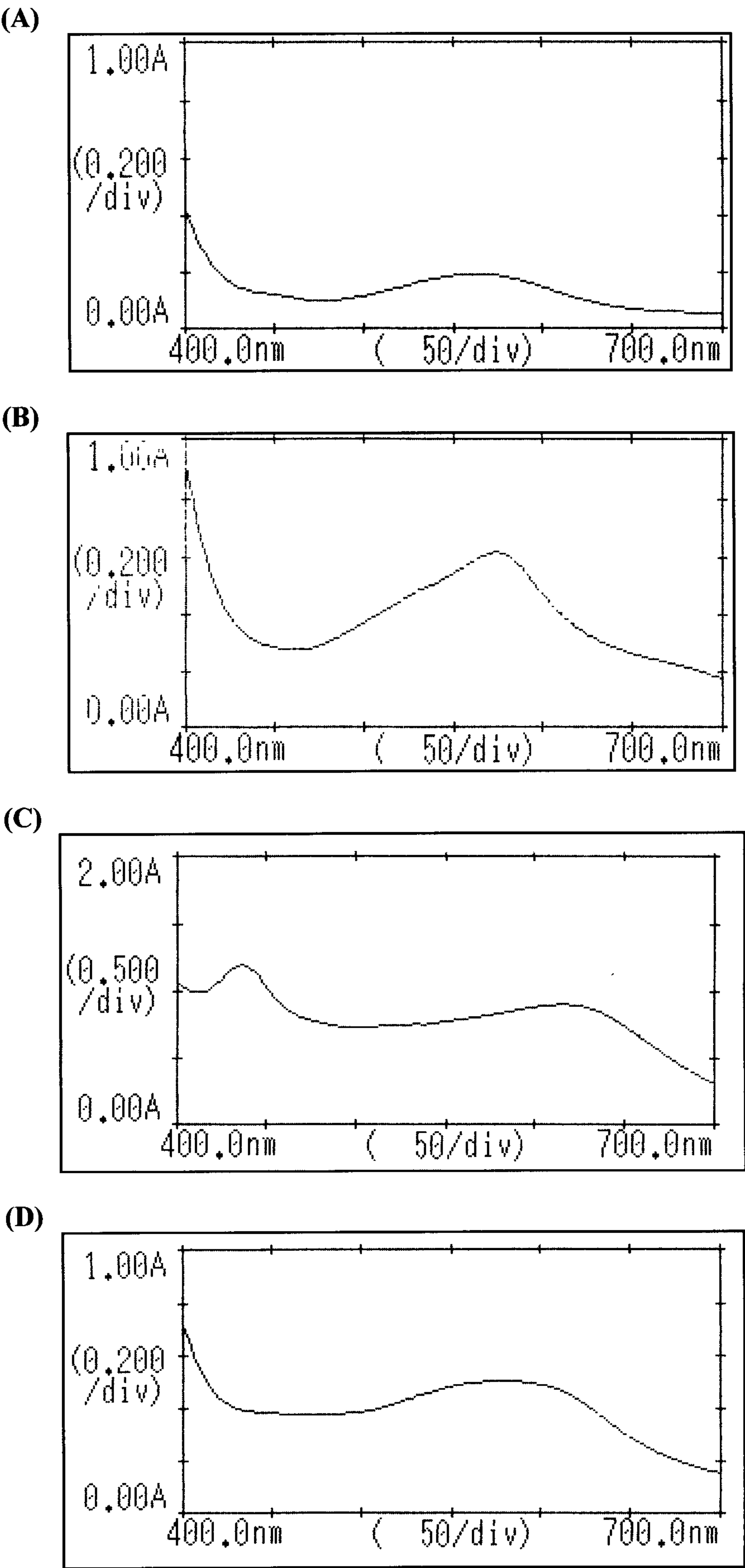




Figure 2.2.5.3. **Absorbance spectra of major neem limonoids subjected to vanillin assay.** Azadirachtin-A, nimbin and salannin were dissolved in volumes of dichloromethane. Vanillin, concentrated sulphuric acid and methanol were added to aliquots of these solutions. This produced coloured mixtures, which were left for 5 minutes to stabilise. Absorbance was then scanned over the wavelength range of 400nm to 700nm, using a UV-VIS spectrophotometer. A blank solution was obtained by substituting the test solution with an equal volume of dichloromethane in the above procedure. **(A)** Absorption spectrum of blank methanol,  $\lambda_{\text{max}} = 569\text{nm}$ . **(B)** Absorption spectrum of azadirachtin-A,  $\lambda_{\text{max}} = 574\text{nm}$ . **(C)** Absorption spectrum of nimbin,  $\lambda_{\text{max}} = 437\text{nm}$  and  $617\text{nm}$ . **(D)** Absorption spectrum of salannin,  $\lambda_{\text{max}} = 574\text{nm}$ .



**Figure 2.2.5.4. The response of the major neem limonoids to the vanillin assay.**

**(A)** Changes in the  $\lambda_{\text{max}}$  wavelength over time. **(B)** Changes in the  $\lambda_{\text{max}}$  absorbance over time. Azadirachtin-A, nimbin and salannin were dissolved in volumes of dichloromethane. Vanillin, concentrated sulphuric acid and methanol were added to aliquots of these solutions. This produced coloured mixtures, which were scanned immediately and during the following 35 minutes over the wavelength range of 400nm to 700nm, using a UV-VIS spectrophotometer. A blank solution was obtained by substituting the test solution with an equal volume of dichloromethane in the above procedure. **(C)** The molar extinction coefficients of the major neem limonoids subjected to vanillin assay. The vanillin assay was conducted using the same methods as described above, with the following modifications. The coloured mixtures were left for 5 minutes to stabilise. Absorption was then measured at the  $\lambda_{\text{max}}$  for each limonoid using an ultraviolet spectrophotometer.

Key: N.A. = Not applicable as peak in absorbance is not present

Nimbin 1<sup>y</sup> = Primary absorption maximum

Nimbin 2<sup>y</sup> = Secondary absorption maximum

Nimbin 3<sup>y</sup> = Tertiary absorption maximum

Peak assignment based on the level of absorption and the time when the peak was first recorded. Peaks with a higher absorbance and occurring earlier received a lower number.

(A)

	$\lambda_{\text{max}}$ (nm) at X minutes					
	0	5	10	15	20	35
Reference	569	569	568	568	568	568
Azadirachtin-A	573	574	574	574	574	574
Nimbin 1 <sup>y</sup>	437	437	435	N.A.	N.A.	N.A.
Nimbin 2 <sup>y</sup>	615	617	614	N.A.	N.A.	N.A.
Nimbin 3 <sup>y</sup>	N.A.	N.A.	519	516	516	517
Salannin	592	574	564	564	559	557

(B)

	Absorbance (AU) at X minutes					
	0	5	10	15	20	35
Reference	0.198	0.201	0.201	0.208	0.210	0.216
Azadirachtin-A	0.637	0.645	0.649	0.657	0.666	0.673
Nimbin 1 <sup>y</sup>	1.376	1.202	1.063	N.A.	N.A.	N.A.
Nimbin 2 <sup>y</sup>	0.931	0.905	0.844	N.A.	N.A.	N.A.
Nimbin 3 <sup>y</sup>	N.A.	N.A.	0.790	0.849	0.876	0.875
Salannin	0.517	0.499	0.490	0.489	0.497	0.502

(C)

	Extinction coefficient at $\lambda_{\text{max}}$ (l.mole <sup>-1</sup> )	$\lambda_{\text{max}}$ (nm)
Azadirachtin-A	1.22 x 10 <sup>3</sup>	574
Nimbin	3.89 x 10 <sup>3</sup>	437
Salannin	2.50 x 10 <sup>3</sup>	574



Figure 2.2.5.5. **Absorbance spectra of crude neem seed extracts (1).** Azadirachtin and related limonoids within semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil were extracted into methanol. The limonoids within the methanol extracts were partitioned into dichloromethane. Vanillin, concentrated sulphuric acid and methanol were added to aliquots of the dichloromethane solutions. This produced coloured mixtures, which were left for 5 minutes to stabilise. Absorbance was then scanned over the wavelength range of 400nm to 700nm, using a UV-VIS spectrophotometer. **(A)** Absorption spectrum of semi-purified azadirachtin,  $\lambda_{\text{max}} = 578\text{nm}$ . **(B)** Absorption spectrum de-fatted neem seed kernel extract,  $\lambda_{\text{max}} = 563\text{nm}$ . **(C)** Absorption spectrum of neem seed oil,  $\lambda_{\text{max}} = 558\text{nm}$ .

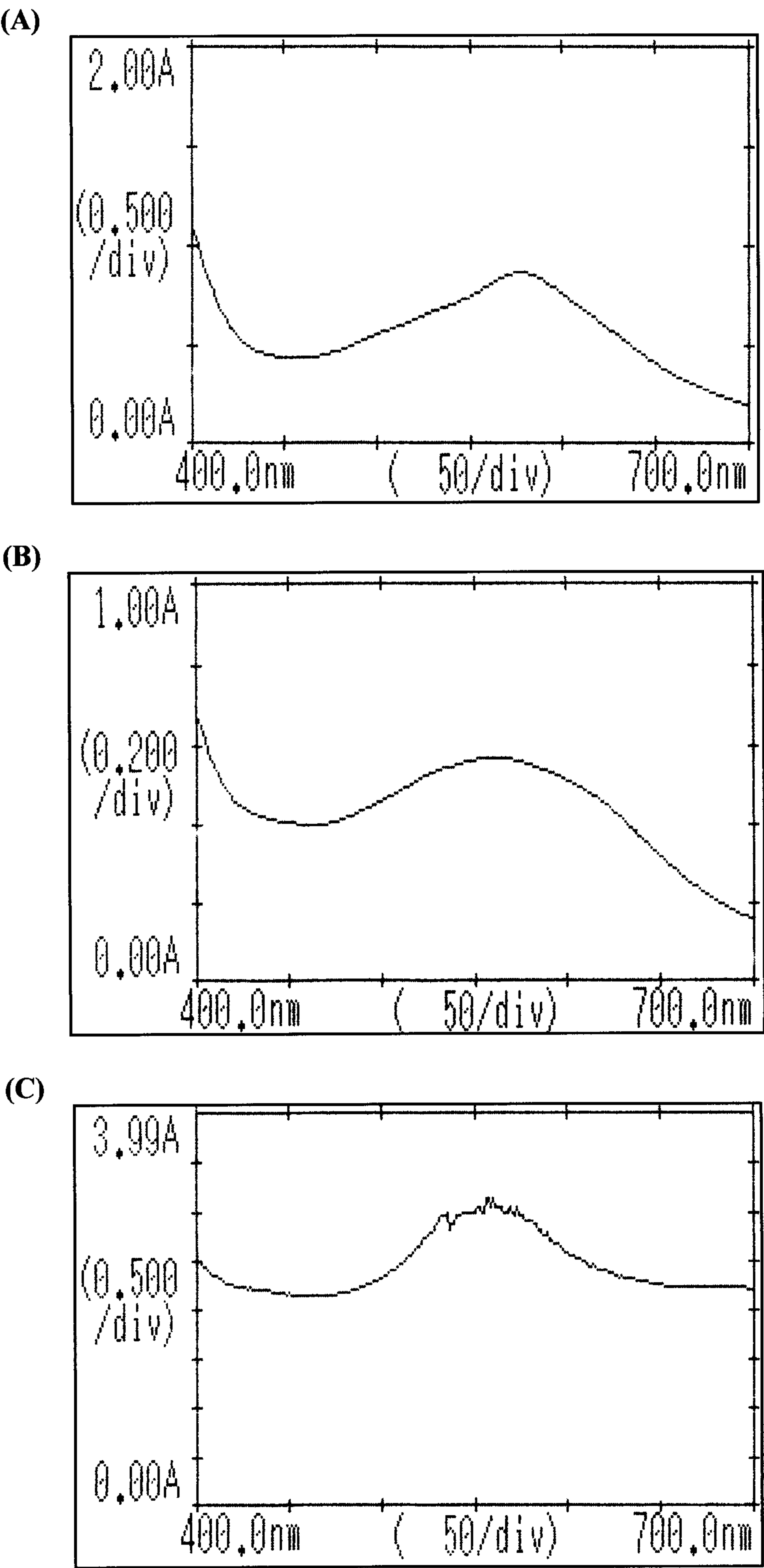
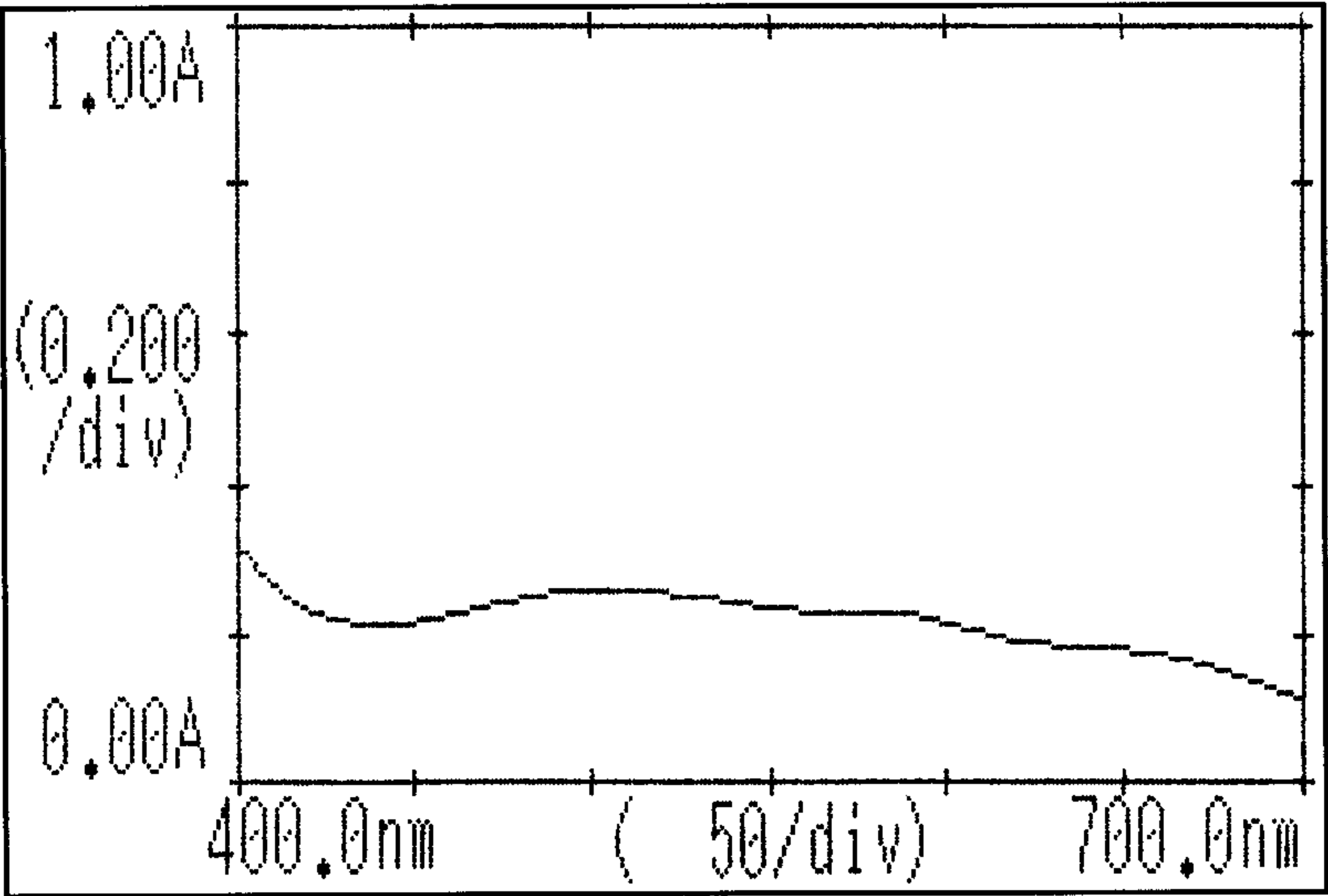
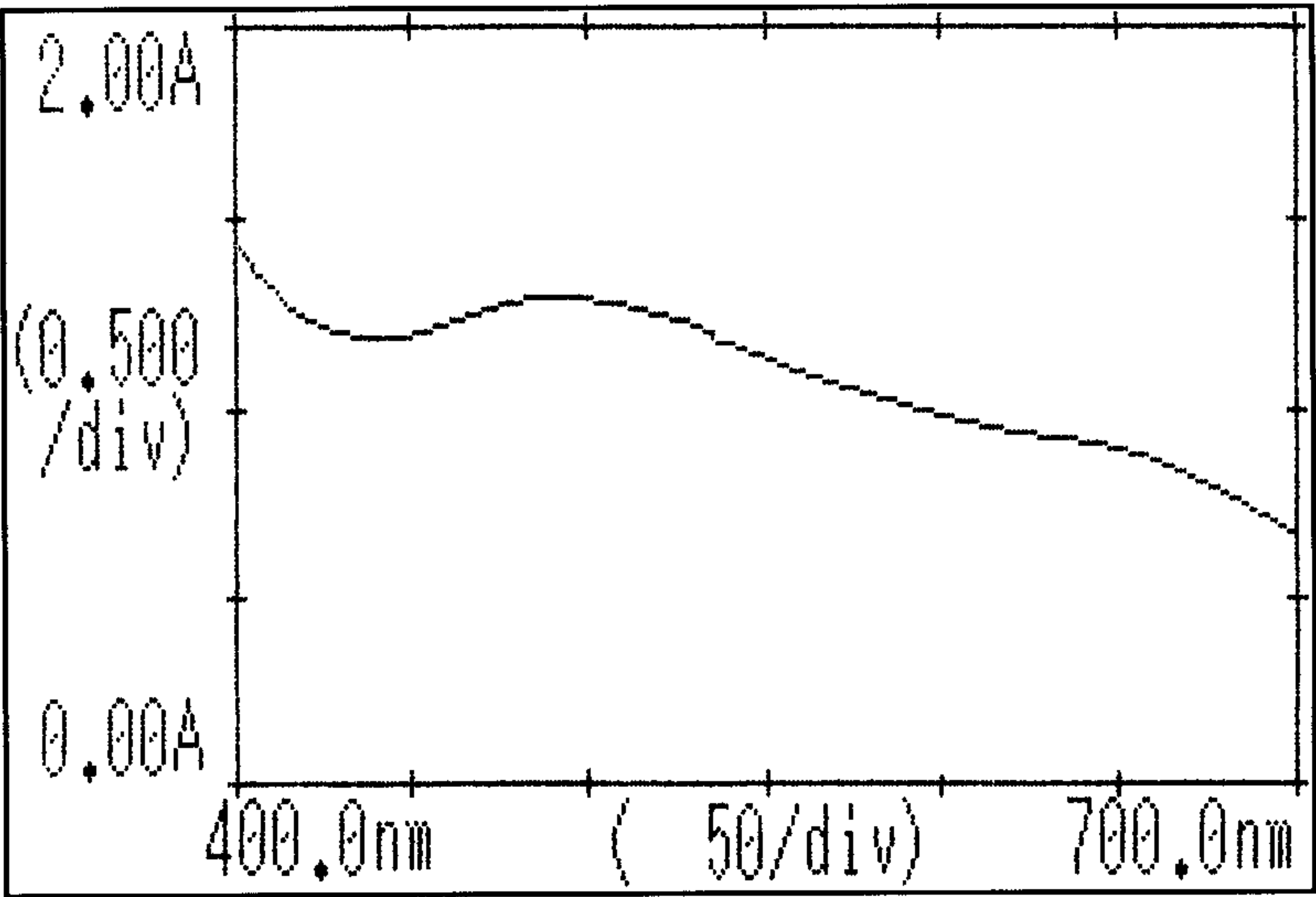


Figure 2.2.5.6. **Absorbance spectra of crude neem seed extracts (2).** The limonoids within semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil were extracted into methanol. Vanillin, concentrated sulphuric acid and methanol were added to aliquots of these solutions. This produced coloured mixtures, which were left for 5 minutes to stabilise. Absorbance was then scanned over the wavelength range of 400nm to 700nm, using a UV-VIS spectrophotometer. **(A)** Absorption spectrum of semi-purified azadirachtin,  $\lambda_{\text{max}} = 503\text{nm} \ \& \ 580\text{nm}$ . **(B)** Absorption spectrum de-fatted neem seed kernel extract,  $\lambda_{\text{max}} = 495\text{nm}$ . **(C)** Absorption spectrum of neem seed oil,  $\lambda_{\text{max}} = 512\text{nm}$ .

(A)



(B)



(C)

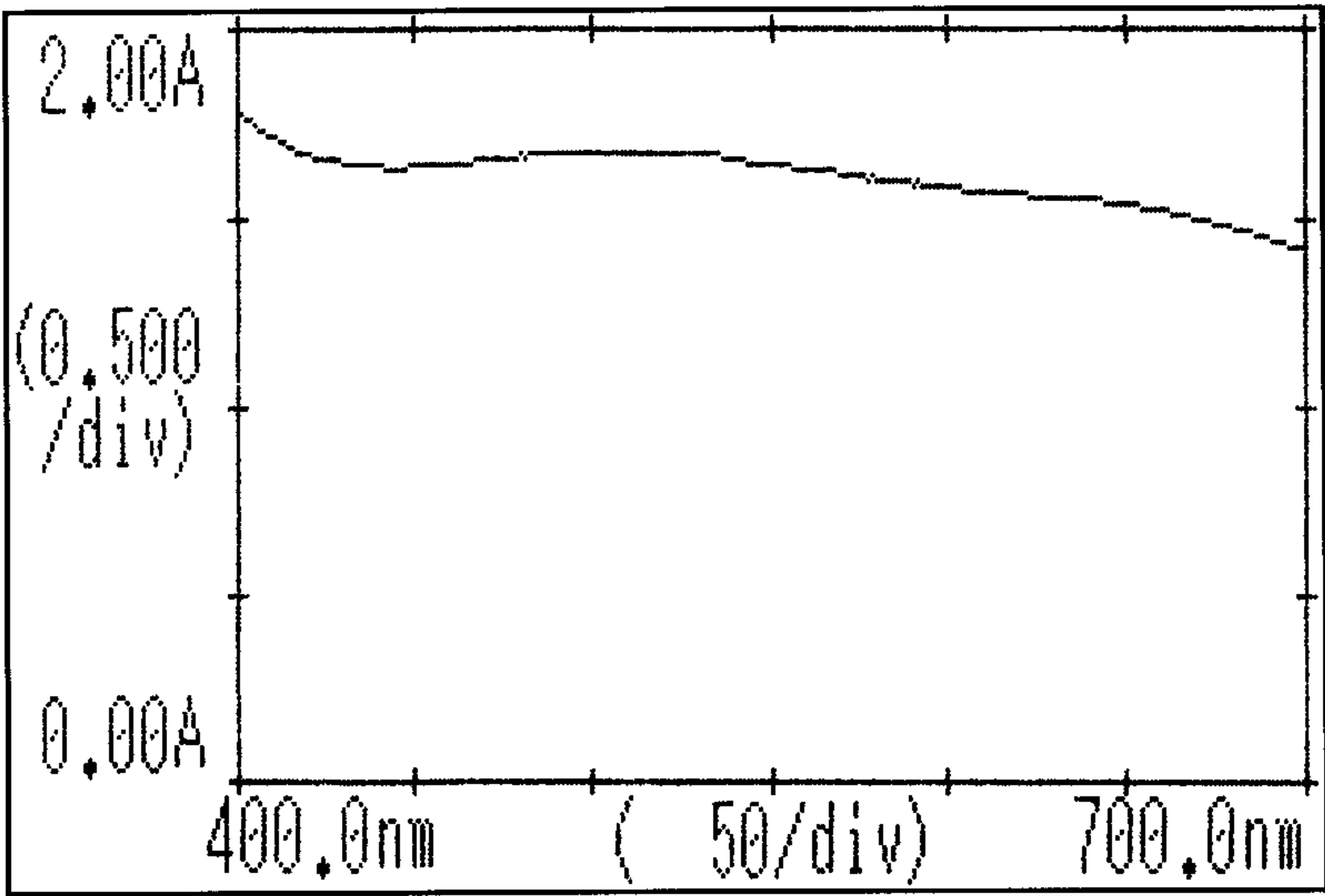
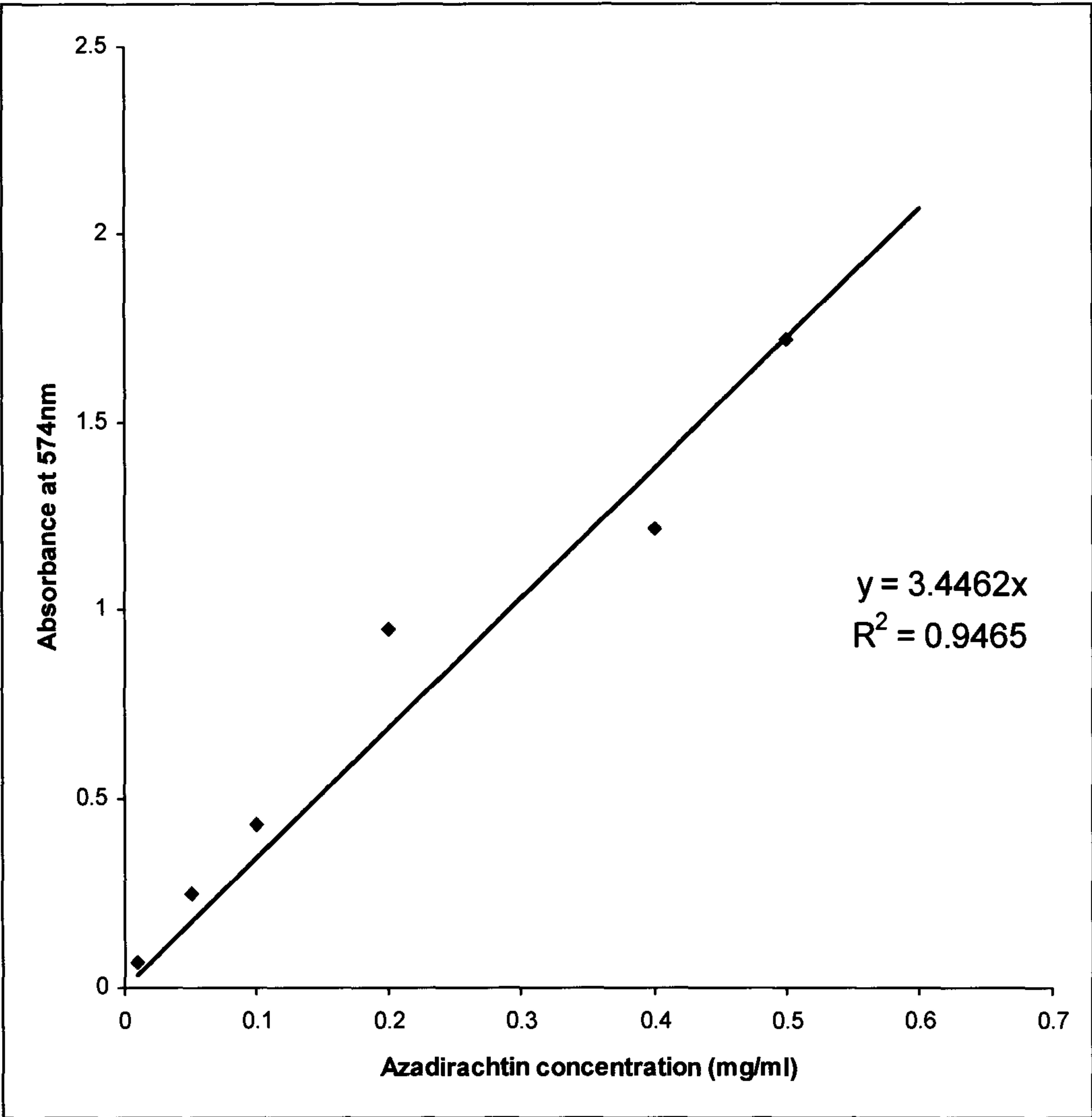




Figure 2.2.5.7. **Azadirachtin-A calibration curve.** Azadirachtin-A (concentration range: 0.01mg/ml-0.5mg/ml) was dissolved into a solution of dichloromethane. Vanillin, concentrated sulphuric acid and methanol were added to aliquots of these solutions. This produced coloured mixtures, which were left for 5 minutes to stabilise. Absorbance was then measured at 574nm using an ultraviolet spectrophotometer.

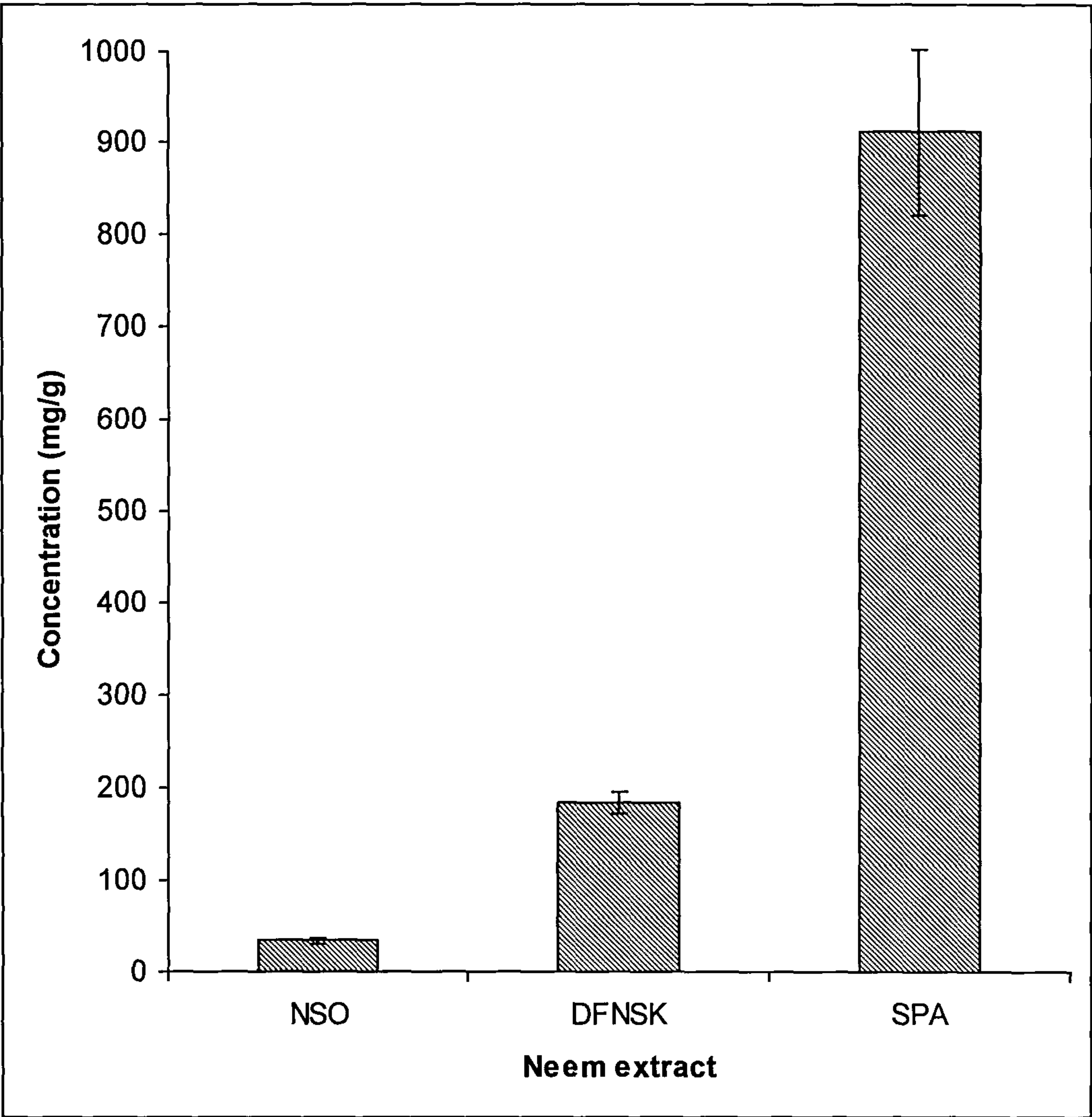


**Figure 2.2.5.8. Concentration of azadirachtin and related limonoids within neem seed extracts, as estimated by the vanillin assay.** In replicates of three, azadirachtin and related limonoids within semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil were extracted into methanol. The limonoids within the methanol extracts were partitioned into dichloromethane. Vanillin, concentrated sulphuric acid and methanol were added to aliquots of the dichloromethane solutions. This produced coloured mixtures, which were left for 5 minutes to stabilise. Absorption was then measured at 574nm using an ultraviolet spectrophotometer. Quantification was achieved by correlating absorbance against azadirachtin concentration on a calibration curve (figure 2.2.5.7.). Vertical lines represent  $\pm$  S.D.

Key: NSO = neem seed oil

DFNSK = de-fatted neem seed extract

SPA = semi-purified azadirachtin





**Figure 2.2.5.9. Concentration of azadirachtin-A, nimbin and salannin within neem seed extracts, as estimated by HPLC.** In replicates of three, the limonoids within semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil were extracted into methanol. Aliquots were analysed using the Beckman HPLC system as described in section 2.2.4.8. (column = C18, solvent system = methanol/water gradient elution). Concentrations were determined by peak area calculations. **(A)** Concentration of azadirachtin-A within crude neem seed extracts. **(B)** Concentration of nimbin within crude neem seed extracts. **(C)** Concentration of salannin within crude neem seed extracts. **(D)** Combined concentration of azadirachtin-A, nimbin and salannin within crude neem seed extracts.

Key: NSO = neem seed oil

DFNSK = de-fatted neem seed extract

SPA = semi-purified azadirachtin

(A)

Neem seed extract	Concentration of azadirachtin-A (mg/g) in extract
NSO	0.39 (± 0.06)
DFNSK	0.87 (± 0.17)
SPA	156.3 (± 3.10)

(B)

Neem seed extract	Concentration of nimbin (mg/g) in extract
NSO	0.06 (± 0.002)
DFNSK	0.12 (± 0.005)
SPA	0.07 (± 0.007)

(C)

Neem seed extract	Concentration of salannin (mg/g) in extract
NSO	0.43 (± 0.012)
DFNSK	0.46 (± 0.066)
SPA	0.13 (± 0.013)

(D)

Neem seed extract	Combined concentration of azadirachtin-A, nimbin and salannin (mg/g) in extract
NSO	0.88 (± 0.074)
DFNSK	1.45 (± 0.241)
SPA	156.5 (± 3.12)

### **2.2.6. Discussion**

As previously detailed, azadirachtin and its related limonoids can be quantified using very few methods. These techniques are normally based on various forms of chromatography and are often criticised for being slow and non-specific (Warthen *et al.*, 1984). Therefore, alternative approaches have been investigated. In particular, Dai *et al.*, (1999) has developed an assay that involves reacting azadirachtin with acidified vanillin solutions to produce coloured complexes. The absorbance of these solutions, at a particular wavelength, corresponding directly to azadirachtin content. This author has suggested that this approach may represent a procedure to quantify azadirachtin within crude mixtures, while not requiring lengthy chromatographic separation.

However, the research of this chapter would suggest that the vanillin assay is not capable of quantifying a single neem limonoid within crude mixtures. Figure 2.2.5.3. shows that azadirachtin-A, nimbin and salannin all reacted with vanillin to produce coloured complexes. The calculated extinction coefficients (figure 2.2.5.4.C.) show that of the limonoids tested, the intensity of absorption would be greatest with salannin and least with azadirachtin-A.

Additionally, the absorption spectra of the pure standards reveal that each limonoid absorbed moderately to strongly throughout the entire visible spectrum (i.e. 400nm-700nm). As a result, there was no wavelength of absorption that was largely unique to a particular limonoid and therefore, could be used as a basis to distinguish the components of mixtures. This observation is supported by figure 2.2.5.5. which show the absorption spectra of semi-purified azadirachtin, de-fatted neem seed kernel

extract and neem seed oil following reaction with the vanillin assay. From an initial observation, it was hoped that each extract would have reacted with vanillin to produce several, distinct absorption maxima. Each  $\lambda_{\max}$  reflecting the absorbance associated with a single neem limonoid. In contrast to this, the crude extracts reacted to give broad peaks in absorption that overlapped with one another.

In addition to the specificity issue mentioned above, other problems of the assay related to the stability of the coloured complexes over time. Figures 2.2.5.4.A. and 2.2.5.4.B. show that for azadirachtin-A, nimbin and salannin, the  $\lambda_{\max}$  of the coloured complex was not stable and drifted in position and/or magnitude as the reaction progressed. This response may influence the accuracy and reproducibility of results.

Methanol was used as a replacement to dichloromethane in the assay to determine if the absorption spectra of the three crude neem seed extracts could be improved. Figure 2.2.5.6. shows that by making this modification the quality of the spectra was considerably reduced. This was suggested, as there were no distinct peaks in absorption formed.

Semi-purified azadirachtin, neem seed oil and de-fatted neem seed kernel extract were quantified using the vanillin assay (figure 2.2.5.8.) and the derived estimations can be compared to those obtained using HPLC (figure 2.2.5.9.). This comparison shows that the vanillin assay reacted not only with azadirachtin-A, nimbin and salannin but also with other related limonoids within extracts. This can be suggested, as estimations using the vanillin assay are far higher than that which can be associated with



azadirachtin-A, nimbin and salannin content alone. As a result, the assay was used to quantify azadirachtin and related limonoids within the neem seed extracts.

The research of this chapter shares a number of similarities with the work of Dai *et al.*, (1999 & 2001b). The absorption spectrum of azadirachtin was almost identical between the two works in terms of shape and  $\lambda_{\text{max}}$ . When studying the response of limonoid mixtures and crude extracts to the assay, Dai *et al.*, also observed band broadening of the absorption maximum. The author associated this effect with interference caused by materials such as phenolics and simple terpenoids, which absorbed at either side of the  $\lambda_{\text{max}}$  of azadirachtin.

The research presented within this chapter would suggest that the vanillin assay is not suitable for directly quantifying azadirachtin or other single limonoid within crude neem extracts, due to specificity limitations. However, the procedure may prove useful in estimating the relative quantities of azadirachtin as well as related neem limonoids within samples during comparative work. Subsequently, for accurate quantification of any single limonoid, analysis by HPLC will still be required.

## **Chapter 3**

### **The Development of a Controlled-Release Granule**

### **3.1. Introduction**

#### **3.1.1. Pesticide formulations**

Pesticides are biologically active in extremely small quantities and therefore, active ingredients must be prepared in a form that is suitable to use while permitting the even distribution of material over the target area (Matthews, 2000). This property can be achieved in a number of ways; for example, the active ingredient can be dissolved or suspended in diluents, such as water or oil (e.g. solutions, suspensions and emulsions), inert solids (e.g. dusts and granules) or as a gas (e.g. aerosols). The field performance of these formulations can be improved through the addition of additives (e.g. wetting agents, anti-evaporants, thickeners and stickers) that enhance the physical properties of droplets, the stability of spray mixtures, ease of application or retention of pesticide on the target (Banks *et al.*, 1990).

Formulation choice is often determined by the chemical characteristics of the active ingredient, regional marketing requirements and the intended pest target (Gardner, 1953 and Matthews, 2000). Most commonly, pesticides are formulated as solutions (active ingredient dissolved in water, organic solvent or oil), emulsifiable concentrates (concentrated oil-in-water emulsions, diluted with water for spraying), wettable powders (finely ground particles of active ingredient, plus wetting and dispersing additives that enable mixing with water), dusts (fine, dry particles, requiring no dilution before use) or granules (pesticide within large, solid particles) (Banks *et al.*, 1990; Barlow, 1985; Matthews, 2000 and Quaglia *et al.*, 2001).

Granular formulations have become increasingly common, as these materials are described as having controlled-release properties, which optimise activity profiles while limiting environmental contact (Barlow, 1985 and Hartley & Graham-Bryce, 1980b).

### 3.1.2. Granular formulations

Granules can be defined as large, discrete, dry particles (typically ranging from 200 $\mu$ m to 2360 $\mu$ m in size) that are insufficiently adhesive to be used in direct applications to plants (Banks *et al.*, 1990; Hartley & Graham-Bryce, 1980b and Matthews, 2000). Therefore, unlike liquid applications that distribute material directly to the pest or its immediate environment, pesticides within granules are indirectly delivered to the target with final distribution of material occurring through local transport systems in a process that is dependant on the physical/chemical properties of the chemical concerned (Barlow, 1985).

Granules are sold ready-to-use and have a considerable convenience advantage for domestic garden use, in that they can be accurately placed using simple dispensers that require little or no cleaning (Banks *et al.*, 1990 and Hartley & Graham-Bryce, 1980b). However, there has been some resistance by farmers to adopt these formulations, due to a lack of suitable products, the requirement of specialised equipment, lack of knowledge regarding optimum application strategy and a lack of uniformity in granule quality (Matthews, 2000).



Granular formulations are produced using a number of techniques, although 3 main methods can be identified. Firstly, an impermeable base of the required particle size can be coated with a layer of pesticide to produce a quick-releasing granule. Secondly, the active ingredient in a suitable solvent can be applied onto particles of a non-disintegrating porous matrix. The solvent is evaporated following pesticide absorption and release is dependent on carrier porosity. Finally, a paste of water, pesticide and powdered carrier can be extruded, cut to appropriate size and dried. Release being dependent on the porosity and disintegration characteristics of the matrix (Barlow, 1985 and Hartley & Graham-Bryce, 1980b).

A number of polymer devices have been used to produce granular formulations, including those from both a synthetic (e.g. poly( $\epsilon$ -caprolactone), polyethylene, poly(vinyl chloride) and poly(acryloyl chloride) and natural origin (e.g. starch, alginates, alkali lignins, clay and clay minerals) (Boydston, 1992; Choudary *et al.*, 1989; Connick, 1982; Cotterill *et al.*, 1996; Cryer & Laskowski, 1998; Nennemann *et al.*, 2001 and Solvay, 1998).

In particular, alginate, starch and poly( $\epsilon$ -caprolactone) have been experimentally investigated as inexpensive, biodegradable matrices for granule production.

### 3.1.3. Alginate granules

Alginic acid is a linear polysaccharide derived from extracts of the Giant Kelp (*Macrocystis pyrifera*) and other brown seaweeds (Phaeophyceae). This polyacid forms gels through complexation with polyvalent cations or polyamines (Connick,

1982; 1988 and Kenaway & Sakran, 1996). These hydrophilic gels have been used to produce a number of herbicide granular formulations and often have additives included such as kaolin clay or linseed oil (Connick *et al.*, 1984; Hussain *et al.*, 1992; Johnson & Pepperman, 1995; Kulkarni *et al.*, 2000; Pepperman *et al.*, 1991 and Pepperman & Kuan, 1995).

#### 3.1.4. Starch granules

Starch (e.g. pearl cornstarch) is a naturally-occurring, biodegradable bipolymer that has been used as an inexpensive, non-toxic matrix for granule formulations (Aminabhavi *et al.*, 1999; Boydston, 1992; Buhler *et al.*, 1994a; Fleming *et al.*, 1992 and Matzinos *et al.*, 2002). Starch-based granules are produced relatively simply in a process involving the dispersion of starch in water, addition of pesticide and cross-linkage of starch chains (Boydston, 1992 and Buhler *et al.*, 1994b). In addition, like alginate granules, release rates have been modified through the addition of additives such as clay or linseed oil (Chafik *et al.*, 1998).

#### 3.1.5. Poly( $\epsilon$ -caprolactone) granules

Poly( $\epsilon$ -caprolactone) is a non-toxic, permeable, biodegradable, aliphatic polyester that is derived from ring opening polymerisation of  $\epsilon$ -caprolactone monomer with alcohol initiators. These products vary in molecular weight from low weight oligomers, for use in the polyurethane industry, up to high molecular weight thermoplastics, which have been used in the controlled-release of drugs, pesticides and fertilisers. Additives such as starch have been blended with poly( $\epsilon$ -caprolactone) to modify mechanical

properties such strength and elongation characteristics. This material has also found uses as synthetic wound dressings and as orthopaedic casts (Bei *et al.*, 2000; Darwis *et al.*, 1998; Matzinos *et al.*, 2002; Qian *et al.*, 2000; Solvay, 1998; Wang *et al.*, 1998; Yavuz *et al.*, 2002).

#### 3.1.6. Controlled-release of pesticides from granules

Granules are often described as having controlled-release properties (Cryer & Laskowski, 1998). Controlled-release can be defined as a technique or method by which active chemicals are made available to a specified target at a rate designed to achieve an intended effect (Kenawy, 1998). This type of formulations has become increasingly popular because they are described as a system by which the efficiency of a pesticide application can be improved (Gan *et al.*, 1994 and Trimnell, 1982).

In contrast to conventional pesticide use, which relies on the repeated application of excessive quantities of material (to offset losses due to breakdown at translocation processes), controlled-release formulations aim at delivering a constant weight of chemical to the local environment of the pest per unit time, while unreleased material remains unavailable in the matrix or carrier. Consequently, a high level of efficiency can be achieved if the rate of release is just sufficient to offset losses while maintaining lethal concentrations (Barlow, 1985; Collins *et al.*, 1973; Collins & Doglia, 1973 and Hermosin *et al.*, 2001).

While the main advantage of controlled-release is the maintenance of effective concentrations for longer durations, these formulations have a number of additional

benefits which include convenience, particularly in handling, improved economics because less active material is required, reduced mammalian toxicity of highly toxic substances, reduced flammability of liquids and reduced phytotoxicity (Hartley & Graham-Bryce, 1980b and Kydonieus, 1980).

Granular formulations are often employed to produce controlled-release effects, however, a number of additional polymeric systems can be used to achieve this behaviour. These are listed on figure 3.1.



Figure 3.1. Categorisation of polymeric systems used for controlled-release (taken from: Kydonieus, 1980).

## I. Physical Systems

### A. Reservoir systems with rate-controlling membrane

1. Microencapsulation
2. Macroencapsulation
3. Membrane systems

### B. Reservoir systems without rate-controlling membrane

1. Hollow fibres
2. Poroplastic® and Sustrelle® Ultramicroporous Cellulose Triacetate
3. Porous polymeric substrates and foams

### C. Monolithic systems

1. Physically dissolved in nonporous, polymeric, or elastomeric matrix
  - a. Nonerodible
  - b. Erodible
  - c. Environmental agent ingression
  - d. Degradable
2. Physically dispersed in nonporous, polymeric, or elastomeric matrix
  - a. Nonerodible
  - b. Erodible
  - c. Environmental agent ingression
  - d. Degradable

### D. Laminated structures

1. Reservoir layer chemically similar to outer control layers
2. Reservoir layer chemically dissimilar to outer control layers

### E. Other physical methods

1. Osmotic pumps
2. Adsorption onto ion-exchange resins

## II. Chemical systems

### A. Chemical erosion of polymer matrix

1. Heterogeneous
2. Homogeneous

### B. Biological erosion of polymer matrix

1. Heterogeneous
2. Homogeneous

### **3.2. Aims**

Neem extracts containing azadirachtin are typically formulated as emulsifiable concentrates for foliar application (see Chapter 1). However, on leaf surfaces azadirachtin exhibits poor insecticidal contact activity and a persistence that is limited primarily by photolysis to 5-7 days (Caboni *et al.*, 2002 and Schmutterer, 1990). Therefore, opportunities exist for the introduction of alternative strategies, which may improve the environmental and biological efficiency of applications. In this respect, the aim of this chapter was to produce a granular controlled-release formulation, containing a neem seed extract that was suitable for application to the soil. Prototype granules based on alginate, starch and poly( $\epsilon$ -caprolactone), with or without additives (kaolin clay or rapeseed oil), and containing different neem seed extracts would be assessed for this purpose.

### **3.3. Materials and methods**

#### **3.3.1. General Materials**

Alginic acid (medium viscosity), starch (corn) and kaolin clay (hydrated aluminium silicate, particle size 0.1 $\mu$ m-4 $\mu$ m) were purchased from Sigma-Aldrich (Poole, Dorset, UK). Vegetable oil (pure rapeseed) was purchased locally. Semi-purified azadirachtin, de-fatted neem seed kernel extract and neem seed oil (described in Chapter 2) were used without further modification.

### **3.4. Experimental**

#### **3.4.1. Production of sodium alginate granules**

A 1.5% sodium alginate solution in distilled water was prepared by gentle heating. After complete cooling, the neem seed extract (semi-purified azadirachtin, de-fatted neem seed kernel extract or neem seed oil) was added at a loading of 5% (w/w) and thoroughly mixed to achieve a uniform dispersion. For formulations with additives, kaolin clay or sunflower oil was added to this neem/alginate solution at 10% (w/w) and thoroughly mixed. The polymer solution was added dropwise through a 3mm orifice into 0.25M  $\text{CaCl}_2$  for a period of 10 minutes with constant stirring. Following the addition, an extra 5 minutes residence time was given, so that the total time spent by the polymer in the gellant solution was 5-15 minutes. Granules were removed from the gellant solution by filtration through a funnel equipped with a coarse fritted disc. The hydrated granules were washed with distilled water, spread out on aluminium foil and air-dried at room temperature for 48-72 hours. Water resulting from syneresis was periodically removed by pipette.

#### **3.4.2. Production of starch-kaolin granules**

A sodium hydroxide solution (2.8ml of 8M) was added under vigorous stirring to a water suspension (100g) containing 14% (w/w) starch and 11% (w/w) kaolin. For formulations containing sunflower oil, this was included at 10% (w/w) into the initial water suspension. To the slurry obtained (pH 13) after 45 minutes, neem seed extract (semi-purified azadirachtin or neem seed oil) was added at 5% (w/w) and dispersed



uniformly throughout. The slurry was added dropwise through a 3mm orifice into 0.25M CaCl<sub>2</sub> for a period of 10 minutes with constant stirring. Following the addition, an extra 5 minutes residence time was given, so that the total time spent by the polymer in the gellant solution was 5-15 minutes. Granules were removed from the gellant solution by filtration through a funnel equipped with a coarse fritted disc. The hydrated granules were washed with distilled water, spread out on aluminium foil and air-dried at room temperature for 48 hours. Water resulting from syneresis was periodically removed by pipette.

#### 3.4.3. Poly( $\epsilon$ -caprolactone) granules

Poly( $\epsilon$ -caprolactone) granules were purchased from the Polymer Processing Research Centre (The Queen's University of Belfast, Belfast) Two batches of granules with different molecular weights (50,000 and 80,000) were prepared. De-fatted neem seed kernel extract was incorporated at 5% (w/w) into granules. The crude neem seed extract was supplied from stocks used during this research.

#### 3.4.4. Measurement of granule size and weight

Replicates of 10 granules were assessed both immediately after removal from the CaCl<sub>2</sub> gellant solution and once completely dried. Hydrated granules were rolled on aluminium foil to remove surface liquid before measurements were taken. Granule size was measured using digiMax precision callipers. Granule weight was recorded using an Ohaus, GA110 electronic balance.

#### 3.4.5. Determination of azadirachtin and related limonoids within granules

Granules (1g) in replicates of three were added to methanol (5ml). Limonoids were extracted using an Ultra Turrax homogeniser (6000rpm for 20 seconds). The resulting extract was centrifuged (3000rpm for 3 minutes) to separate particulate material from the methanol. This methanol phase was collected. The extraction procedure was repeated on the partially extracted granules a further 2 times. Extracts were pooled and filtered using a syringe fitted with a 0.2µm syringe filter. A volume of the extract was removed and suitably diluted into methanol. An aliquot of this dilution was analysed by reversed-phase HPLC using the conditions described in section 3.4.10. An additional aliquot of the undiluted methanol extract was added to distilled water (1ml) and the limonoids within partitioned into dichloromethane (1ml) by vortex mixing for 20 seconds. The mixture was spun by centrifuge (3000rpm for 1 minute) into distinct phases. The hypophase was collected by pipette and the limonoids remaining within the aqueous hyperphase were partitioned into fresh dichloromethane a further two times using this method. Hypophase collections were pooled and evaluated for azadirachtin and related limonoid content using the vanillin assay described in Chapter 2. Extraction of limonoids from poly(ε-caprolactone) granules was not possible. Quantification of poly(ε-caprolactone) formulations was estimated based on a granule loading of de-fatted neem seed kernel extract at 5% (w/w).

#### 3.4.6. The release of azadirachtin and related limonoids from granular formulations

Batches of granules (2g) in replicates of four were placed into separate 50ml conical flasks, each containing distilled water (12ml at pH 6.0) and sodium azide (0.01%).

These were sealed, placed on an oscillating shaker at  $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$  and maintained in the dark. Samples were taken during a 28-day period at, 0, 1, 2, 4, 8, 24, 48, 96, 168, 264, 336, 504 and 672 hours. At these sampling points the total quantity of water within the flasks was collected and replaced with a fresh volume (12ml at pH 6.0) containing sodium azide (0.01%). Flasks were again sealed and maintained under the same conditions until the next sampling point, when the process was repeated. Collected samples were filtered using a syringe fitted with a  $0.2\mu\text{m}$  syringe filter. An aliquot was removed and analysed by reversed-phase HPLC using the conditions described in section 3.4.10. An additional aliquot was taken and the limonoids within partitioned into dichloromethane using the methods described in section 3.4.5. Azadirachtin and related limonoid content was estimated using the vanillin assay described in Chapter 2.

#### 3.4.7. Swelling study of granules

At 0, 1, 2, 4, 8, 24, 48, 96, 168, 264, 336, 504 and 672 hours, single granules in replicates of 5 were removed from each of the batches being maintained in water (see section 3.4.6.). These were rolled on aluminium foil to remove surface liquid. Granule size and weight was measured using the apparatus described in section 3.4.4. Following measurements, granules were returned to the appropriate flasks.

#### 3.4.8. The breakdown of azadirachtin and related limonoids within granular formulations after 28 days immersed in water

Following the final granule release assessment at 672 hours (see section 3.4.6.), granules were removed from the flasks and air-dried on aluminium foil. Water resulting from syneresis was periodically removed by pipette. Batches of dried granules (0.5g), in replicates of three were taken. Azadirachtin and related limonoids were extracted and quantified using the methods described in section 3.4.5. Percentage breakdown was estimated by comparing the percentage of azadirachtin and related limonoids released from granules to the percentage of azadirachtin and related limonoids remaining within granules. Unaccounted material was assumed to have degraded.

#### 3.4.9. The shelf life of sodium alginate granular formulations

Batches of sodium alginate granules (10g) containing semi-purified azadirachtin were placed into separate glass containers and sealed. Two batches were stored in the dark and another two batches kept at a position exposed to natural, direct sunlight. Both sets were maintained at  $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$ . Granules (0.5g) in replicates of three were removed at 0, 30, 90, 150, 180, and 240 days. Azadirachtin and related limonoids were extracted and quantified using the methods described in section 3.4.5. Azadirachtin and related limonoids were estimated using the vanillin assay described in Chapter 2. The half-life was calculated from plots of natural log (percentage of material remaining) against time.



#### 3.4.10. Analytical high performance liquid chromatography using the Beckman HPLC system

Analytical reversed-phase HPLC was carried out with a Beckman HPLC system. Aliquots were injected onto a C18 column (Waters, 4.6 x 250mm, particle size: 5µm) using a Beckman (210A) injector fitted with a 200µl injection loop. Materials eluting from the column were detected using a Beckman (166), system gold, programmable detector, set at 217nm that was equipped with a Beckman (406), system gold, analogue interface module. Detected peaks were estimated valley to valley and retention times were recorded using a NEC PC-8300 module linked to a Servogor chart recorder. The solvent system consisted of methanol/water run in a gradient elution. The ratio of methanol to water was increased from 60% to 100% over a 10-minute period and returned from 100% to 60% over a 2-minute period. There was a flow rate of 1ml/min, at an average pressure of 207 bar. Pressure was controlled through a Beckman (110B) solvent delivery module. Materials eluting from the column were identified through retention time and comparison with known standards.

### **3.5. Results**

#### **3.5.1. Granule production**

Granular neem formulations, based on sodium alginate, starch-kaolin and poly( $\epsilon$ -caprolactone), with or without additives were successfully prepared. These formulations varied in colour, size and weight.

Sodium alginate granules were produced that contained de-fatted neem seed kernel extract, neem seed oil and semi-purified azadirachtin. In addition, kaolin clay and rapeseed oil were incorporated into formulations containing semi-purified azadirachtin (figures 3.5.1. & 3.5.2.). Hydrated granules were tough, elastic and spherical in shape, ranging from 4.3mm-5.2mm in diameter and 46.2mg-77.9mg in weight (figure 3.5.5.). Dried formulations were hard, of a spherical shape and varied between 1.9mm-2.9mm in diameter and 3.8mg-14.0mg in weight (figure 3.5.6.). Only dried granule diameter was significantly ( $p < 0.05$ ) changed by the addition of kaolin clay or rapeseed oil to formulations.

Starch-kaolin granules were prepared that contained neem seed oil and semi-purified azadirachtin. In addition, rapeseed oil was incorporated into formulations containing semi-purified azadirachtin (figure 3.5.3.). Hydrated granules were spherical, soft and fragile and tended to aggregate together. In this form, granules ranged from 4.4mm-5.1mm in diameter and 41mg-49mg in weight (figure 3.5.5.). Dried formulations were brittle deformed spheres, which had flattened sides with surface cavities. These granules varied between 2.7mm-3.5mm in diameter and 13.1mg-19.8mg in weight

(figure 3.5.6.). The addition of rapeseed oil did not significantly ( $p>0.05$ ) change granule size or weight.

Poly( $\epsilon$ -caprolactone) granules were prepared that contained de-fatted neem seed kernel extract (figure 3.5.4.). Granules were elongated, extremely hard and ranged from 3.3mm-3.6mm in diameter and 16.8 mg-31.4mg in weight (figure 3.5.6.).

The concentration of azadirachtin and related limonoids within granular formulations was variable (figure 3.5.7.). Concentrations within sodium alginate granules ranged from 11mg/g in granules containing neem seed oil to 600mg/g in granules containing semi-purified azadirachtin only. Starch-kaolin granules contained 8mg/g when neem seed oil was used in formulations to 144mg/g when semi-purified azadirachtin was solely incorporated into the granule matrix. Poly( $\epsilon$ -caprolactone) granules contained an estimated 9.2mg/g of azadirachtin and related limonoids.

Figure 3.5.1. **Photographs of sodium alginate granules (1).** A sodium alginate solution containing neem seed extract was added dropwise into 0.25M  $\text{CaCl}_2$  to produce gelatinous beads. These were removed from the gellant solution by filtration. Granules were air-dried to produce dry formulations. **(A)** Granules immediately after removal from gellant solution. **(B)** Completely dried granules. Pictures are shown with a millimetre scale.



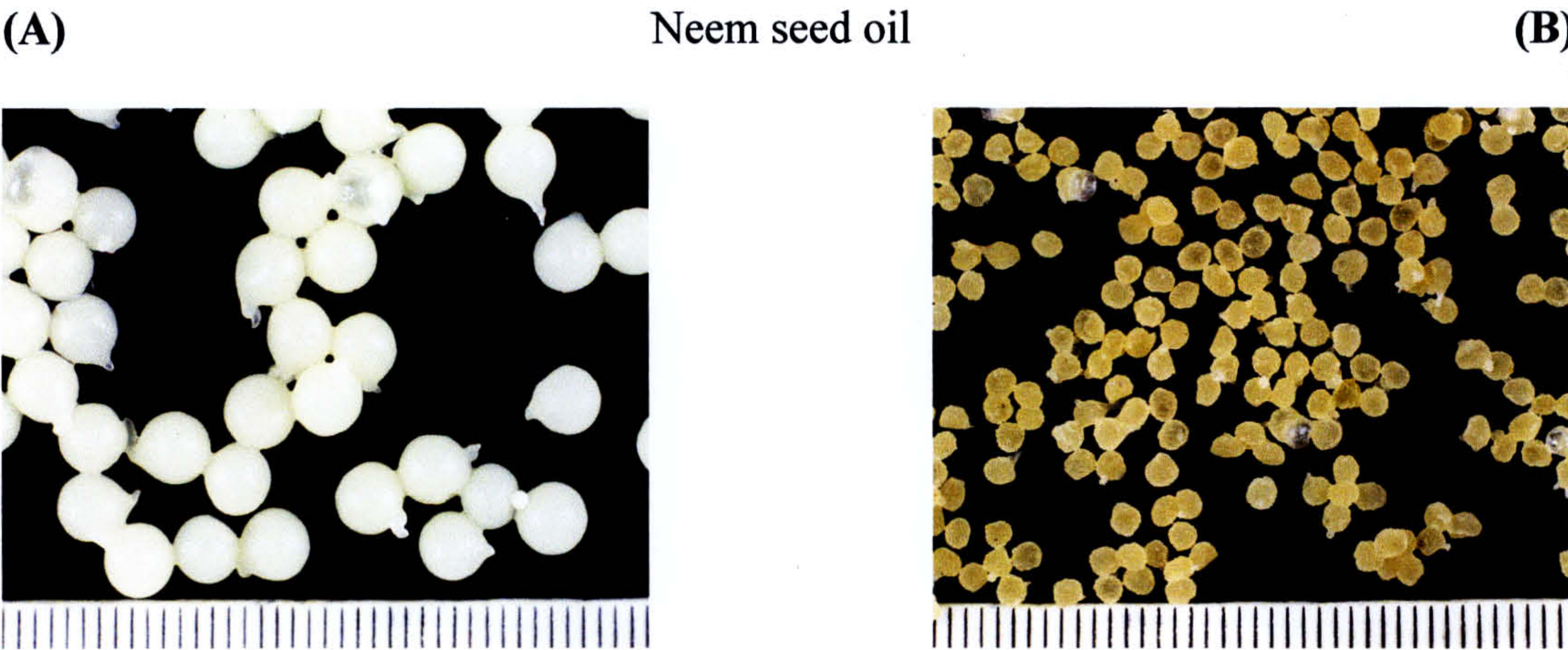
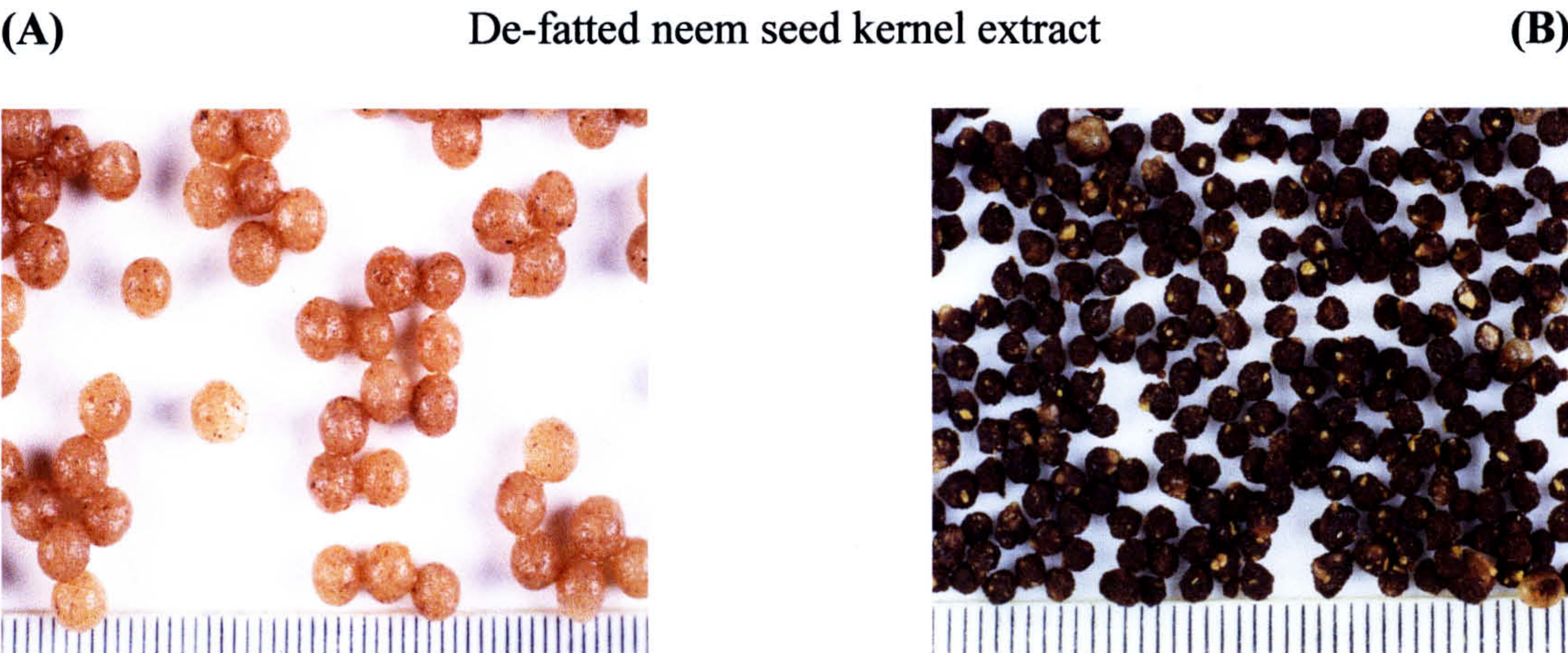
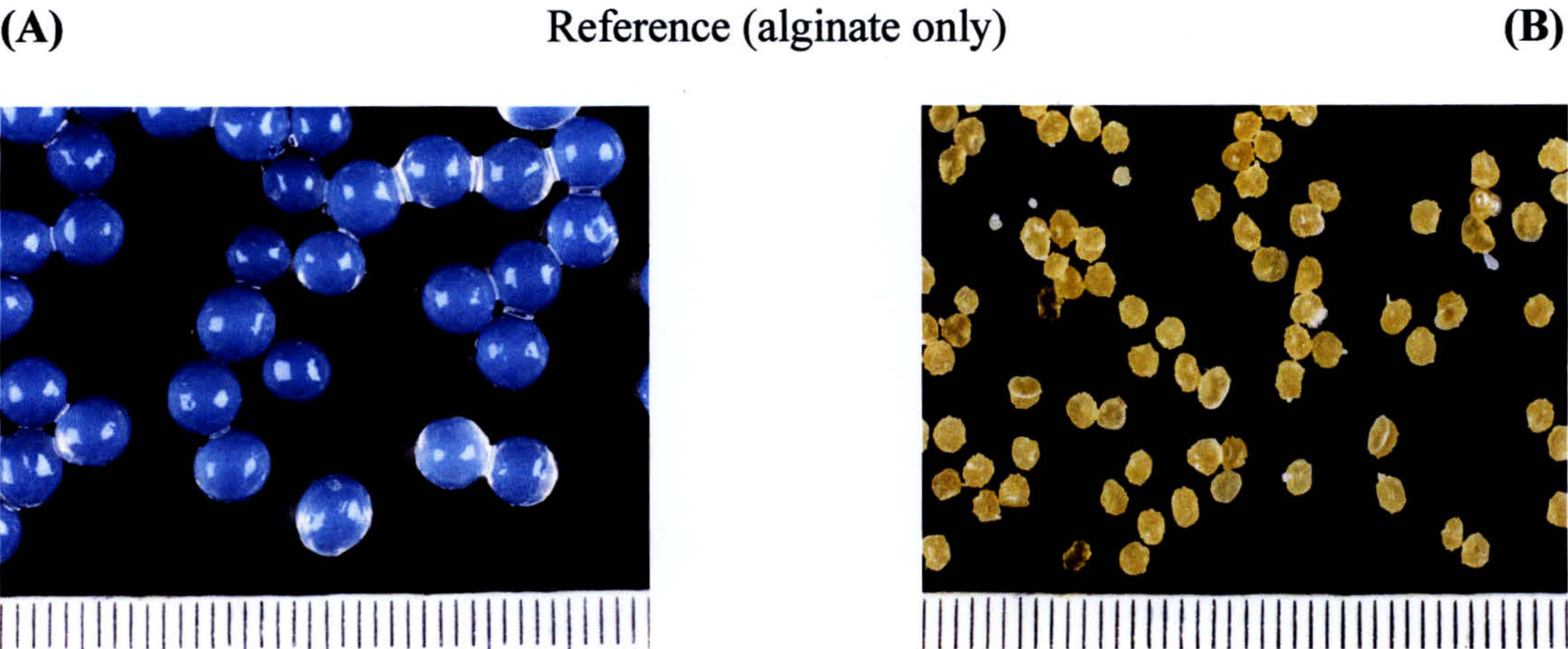




Figure 3.5.2. **Photographs of sodium alginate granules (2).** A sodium alginate solution containing semi-purified azadirachtin, with or without additive, was added dropwise into 0.25M CaCl<sub>2</sub> to produce gelatinous beads. These were removed from the gellant solution by filtration. Granules were air-dried to produce dry formulations. **(A)** Granules immediately after removal from gellant solution. **(B)** Completely dried granules. Pictures are shown with a millimetre scale.



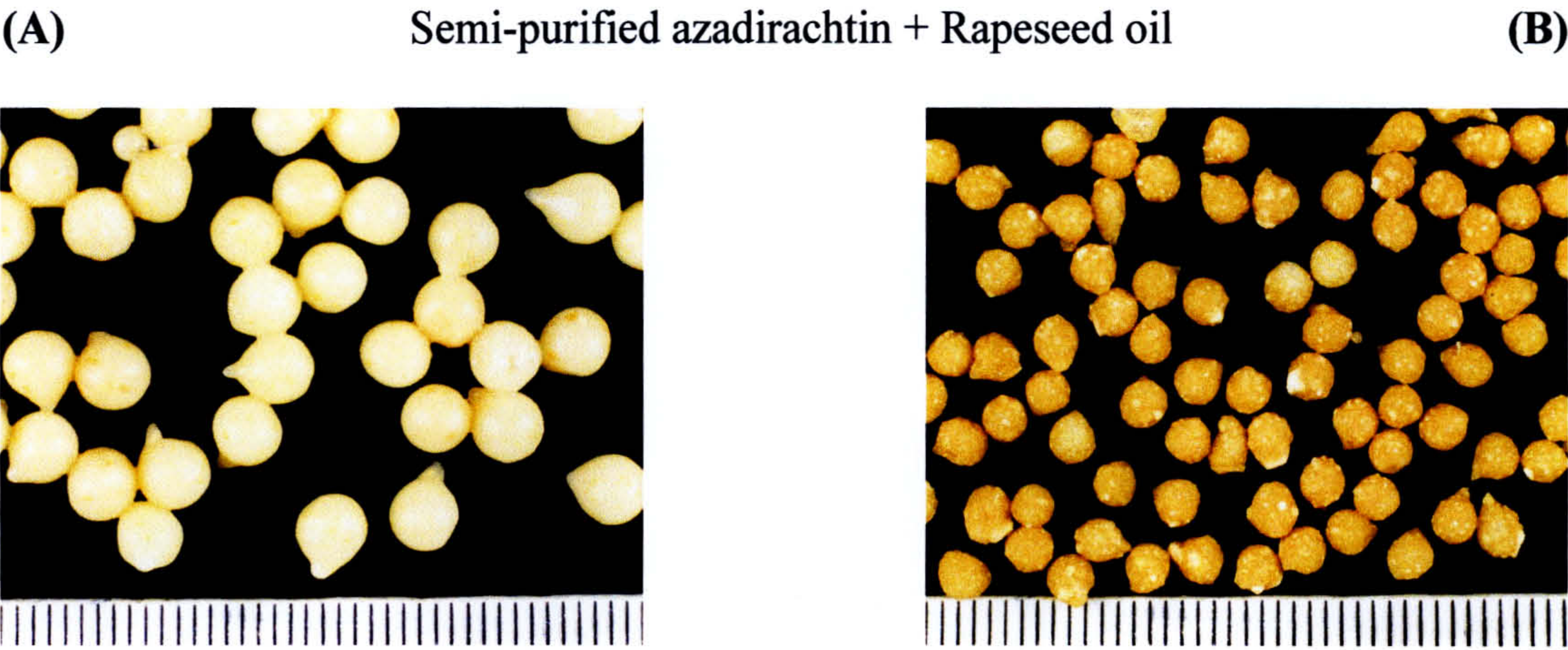
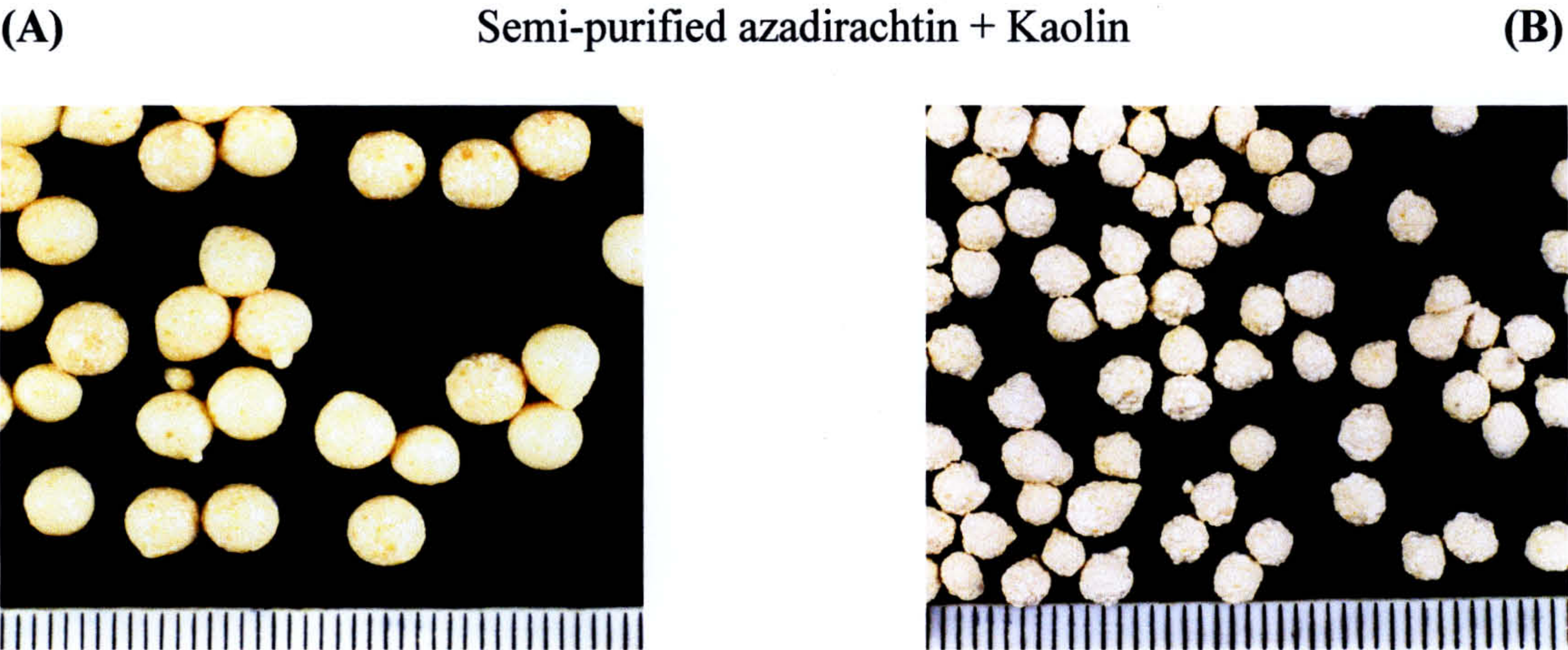
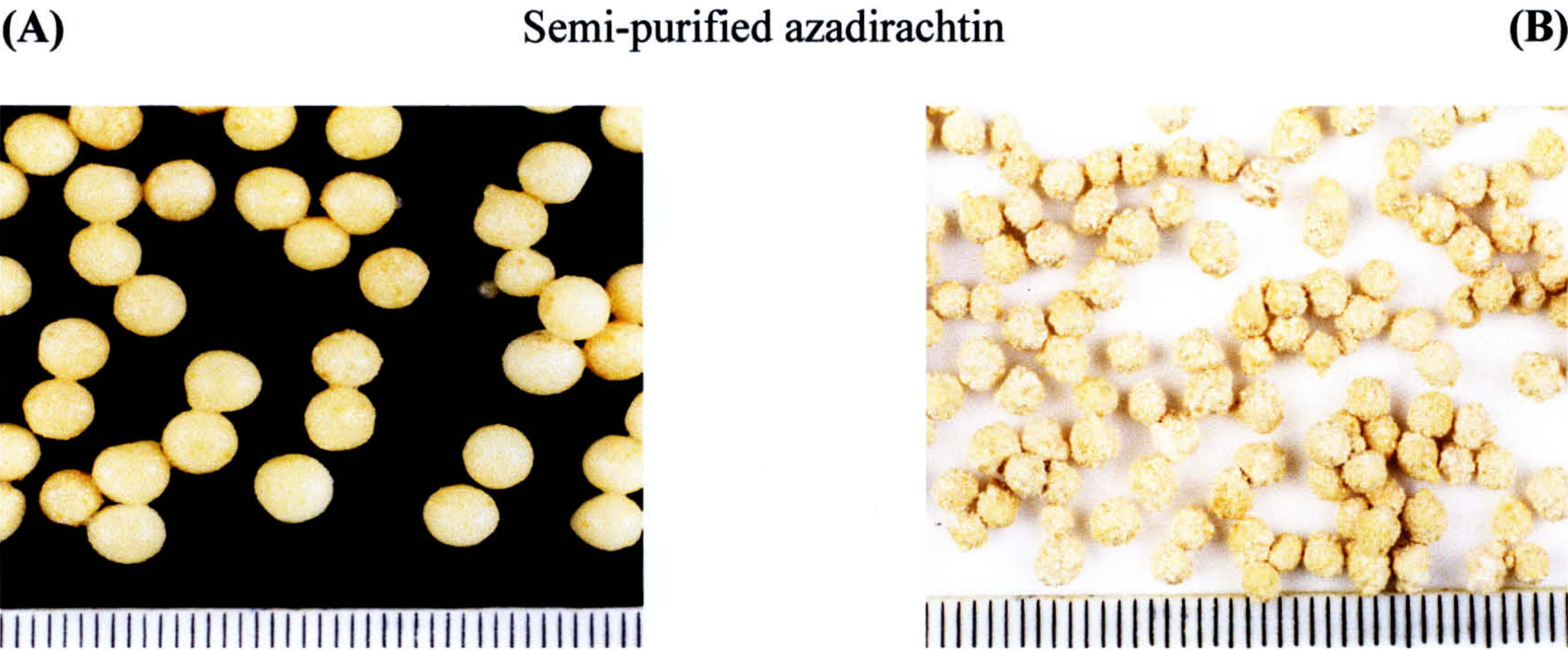




Figure 3.5.3. **Photographs of starch-kaolin granules.** A starch-kaolin slurry containing neem seed extract, with or without additive, was added dropwise into 0.25M CaCl<sub>2</sub> to produce gelatinous beads. These were removed from the gellant solution by filtration. Granules were air-dried to produce dry formulations. **(A)** Granules immediately after removal from gellant solution. **(B)** Completely dried granules. Pictures are shown with a millimetre scale.



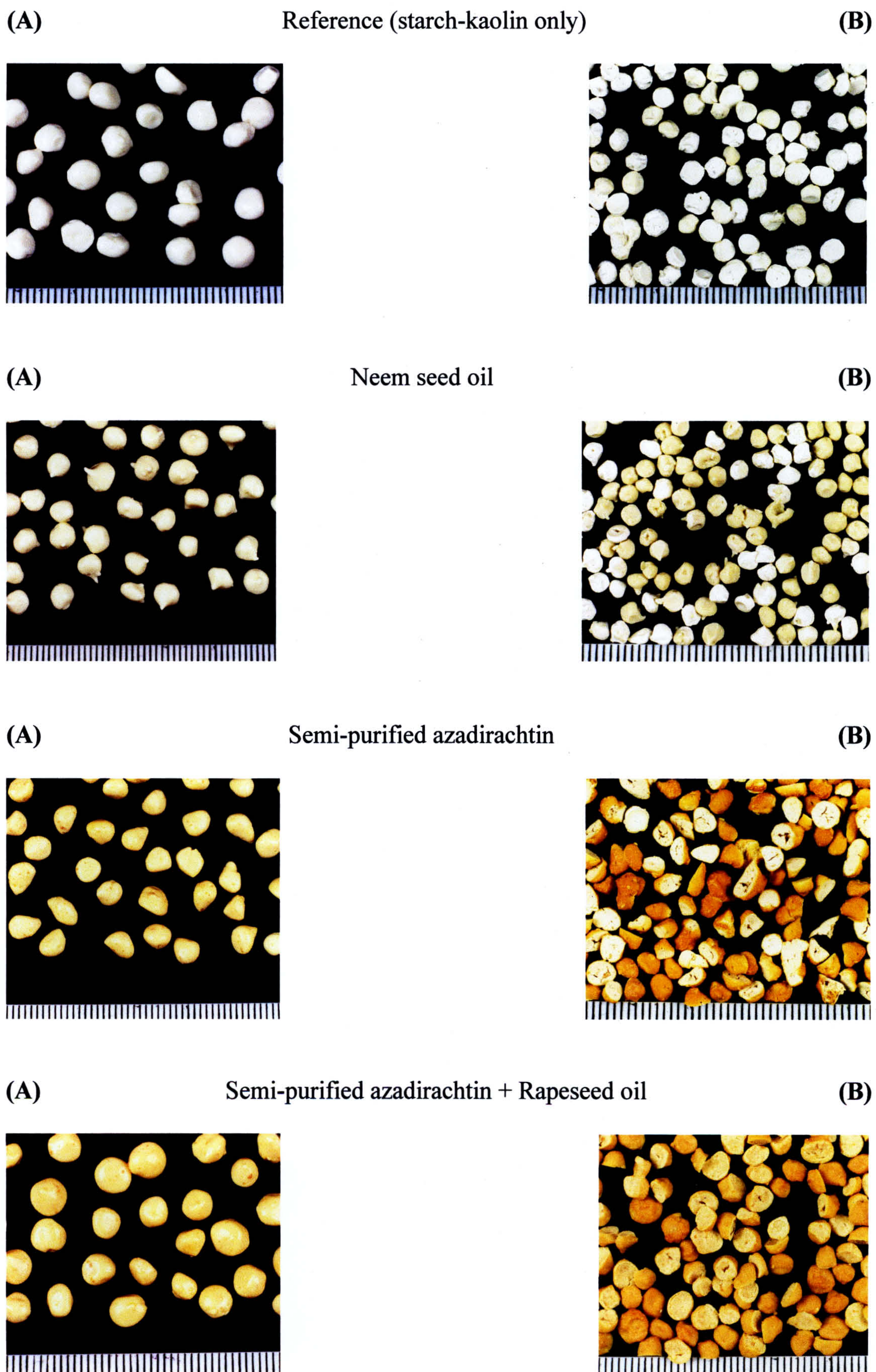
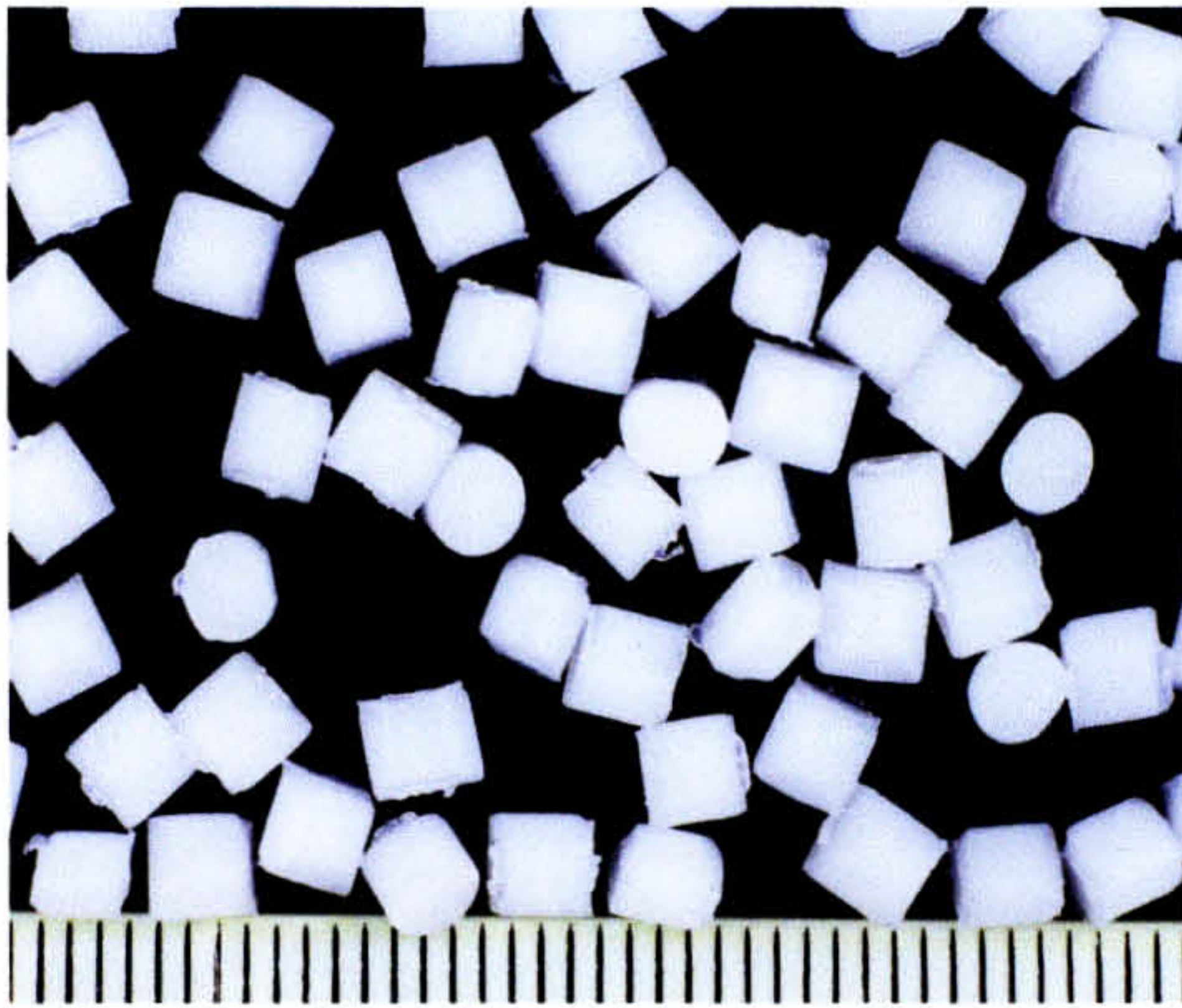




Figure 3.5.4. **Photographs of poly( $\epsilon$ -caprolactone) granules.** Granules were purchased from the Polymer Processing Research Centre (The Queen's University of Belfast, Belfast) and contained de-fatted neem seed kernel extract at a loading of 5% (w/w). Two batches of granules with different molecular weights were prepared. **(A)** Molecular weight 50,000. **(B)** Molecular weight 80,000. Pictures are shown with a millimetre scale.



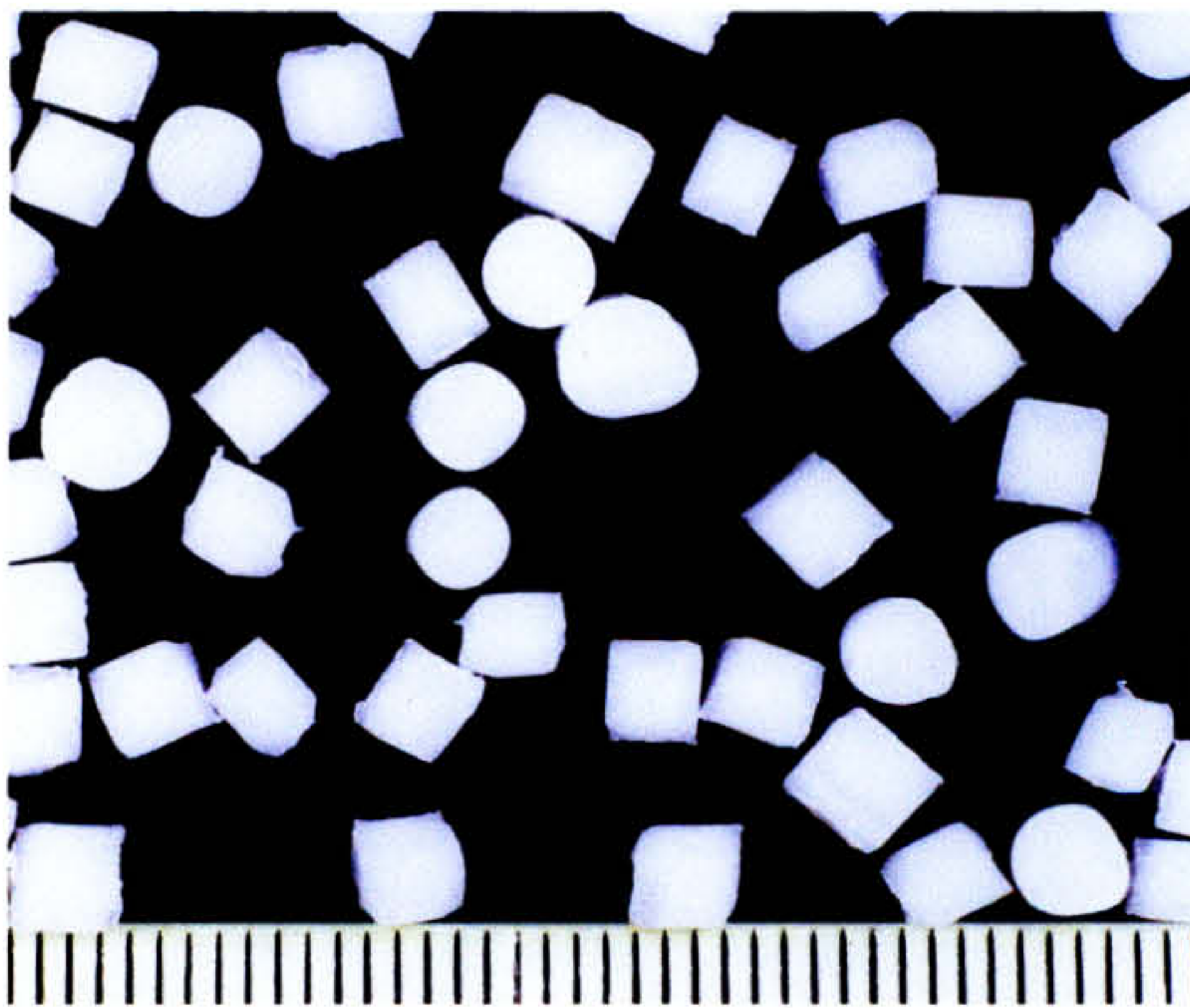
(A) Reference



De-fatted neem seed kernel extract



(B) Reference

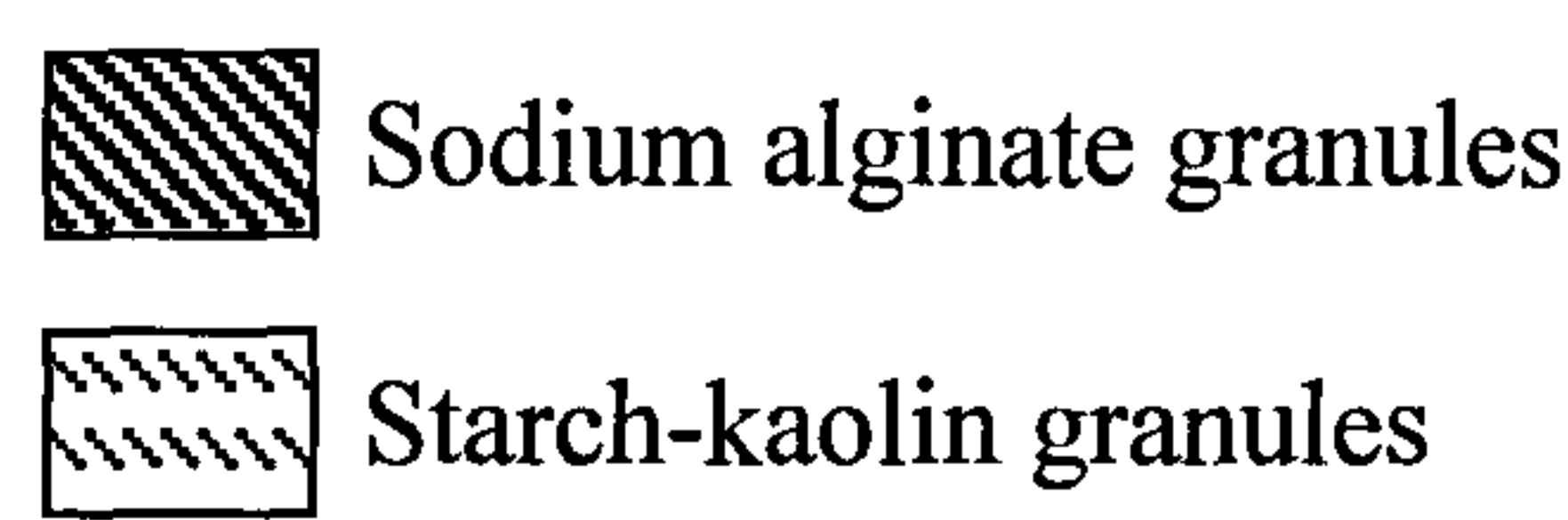


De-fatted neem seed kernel extract





Figure 3.5.5. **Hydrated granule sizes and weights.** After removal from the CaCl<sub>2</sub> gellant solution, granules in replicates of 10 were rinsed with distilled water, rolled on aluminium foil and measured. Poly( $\epsilon$ -caprolactone) granules were not produced in hydrated form. **(A)** Hydrated granule diameter. **(B)** Hydrated granule weight. The code terminology used is detailed below. Vertical lines represent  $\pm$  S.D.



Code	Neem material	Additive	Matrix
REF/SA	None	None	Sodium alginate
DFNSK/SA	De-fatted neem seed kernel extract	None	Sodium alginate
NSO/SA	Neem seed oil	None	Sodium alginate
SPA/SA	Semi-purified azadirachtin	None	Sodium alginate
SPA/KA/SA	Semi-purified azadirachtin	Kaolin	Sodium alginate
SPA/OIL/SA	Semi-purified azadirachtin	Rapeseed oil	Sodium alginate
REF/ST-KA	None	None	Starch-kaolin
NSO/ST-KA	Neem seed oil	None	Starch-kaolin
SPA/OIL/ST-KA	Semi-purified azadirachtin	Rapeseed oil	Starch-kaolin
SPA/ST-KA	Semi-purified azadirachtin	None	Starch-kaolin



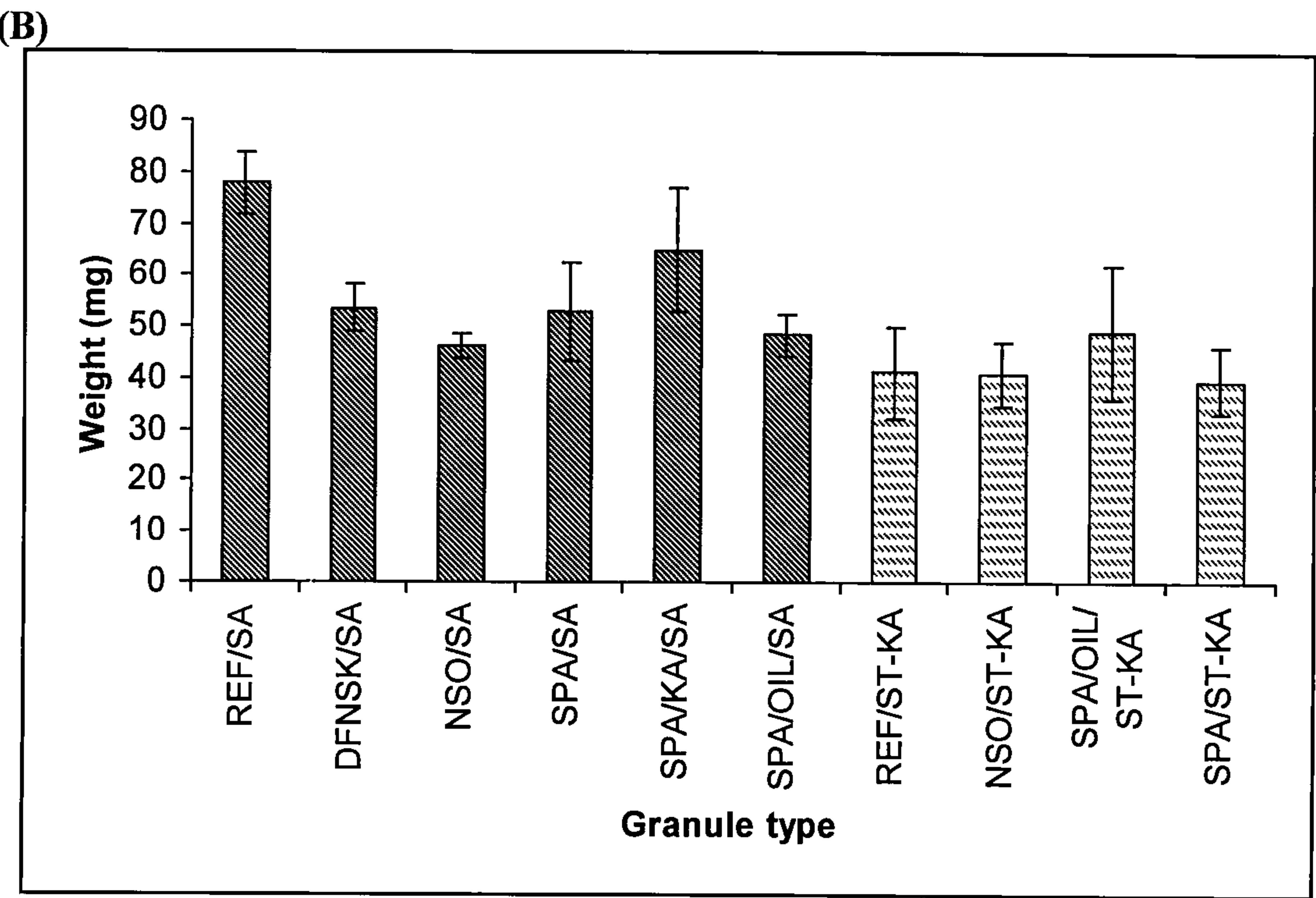
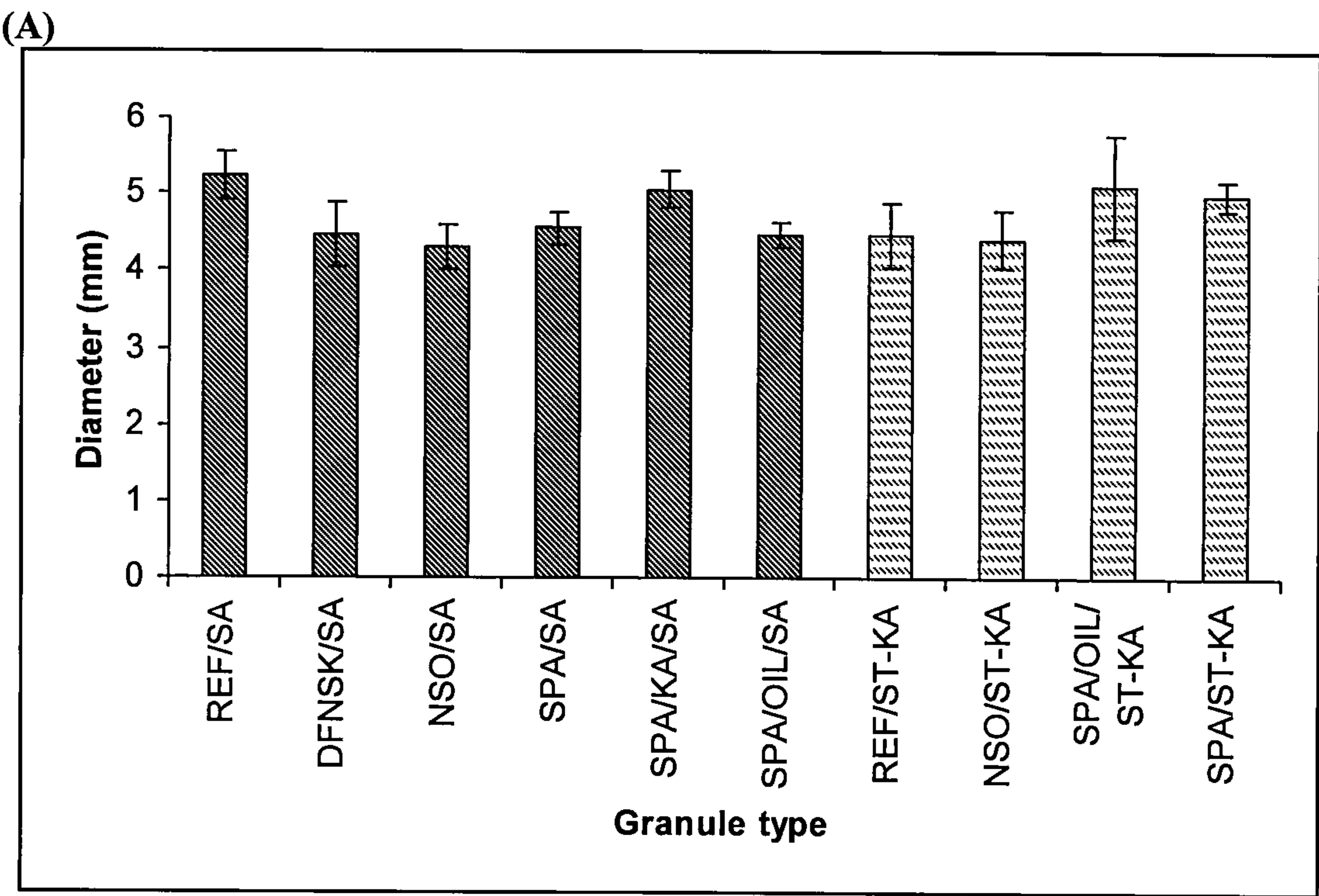
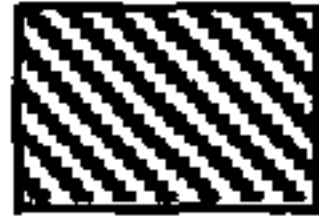


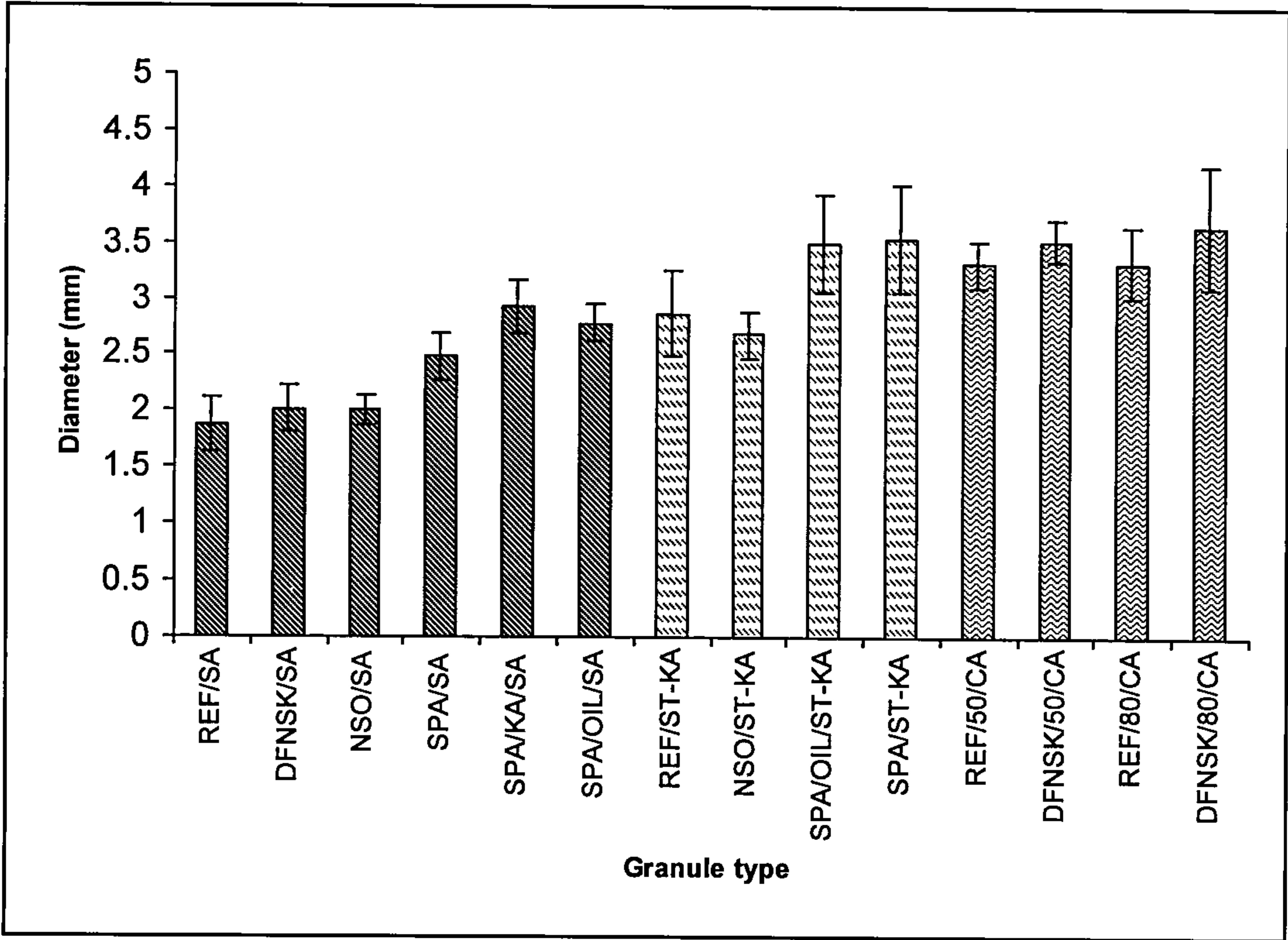


Figure 3.5.6. **Dried granule sizes and weights.** Granules after complete drying were measured in replicates of ten. **(A)** Dried granule diameter. **(B)** Dried granule weight. The code terminology used is detailed below. Vertical lines represent  $\pm$  S.D.

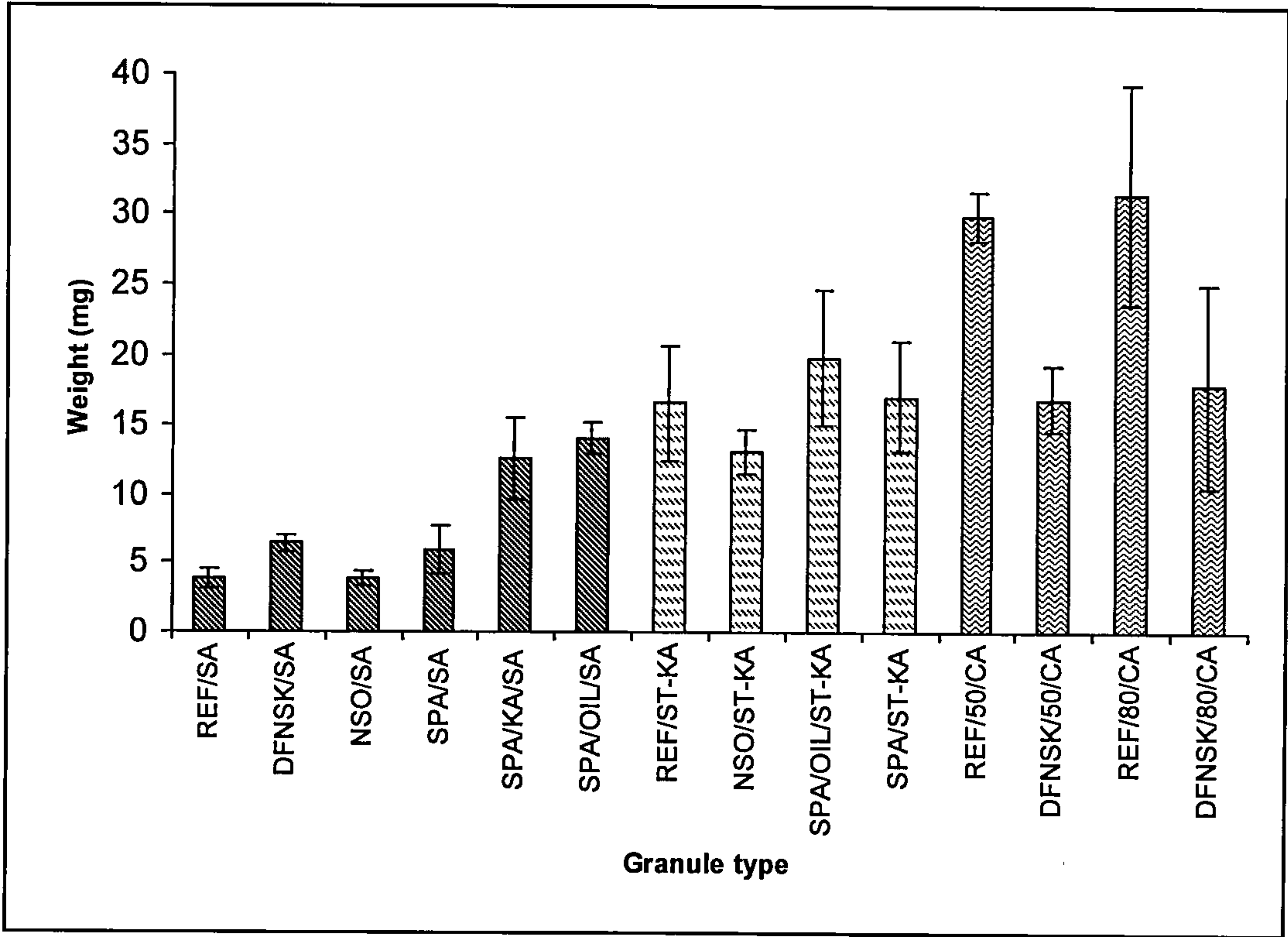
-  Sodium alginate granules
-  Starch-kaolin granules
-  Poly(ε-caprolactone) granules

Code	Neem material	Additive	Matrix
REF/SA	None	None	Sodium alginate
DFNSK/SA	De-fatted neem seed kernel extract	None	Sodium alginate
NSO/SA	Neem seed oil	None	Sodium alginate
SPA/SA	Semi-purified azadirachtin	None	Sodium alginate
SPA/KA/SA	Semi-purified azadirachtin	Kaolin	Sodium alginate
SPA/OIL/SA	Semi-purified azadirachtin	Rapeseed oil	Sodium alginate
REF/ST-KA	None	None	Starch-kaolin
NSO/ST-KA	Neem seed oil	None	Starch-kaolin
SPA/OIL/ST-KA	Semi-purified azadirachtin	Rapeseed oil	Starch-kaolin
SPA/ST-KA	Semi-purified azadirachtin	None	Starch-kaolin
REF/50/CA	None	None	Poly(ε-caprolactone) Mw 50,000
DFNSK/50/CA	De-fatted neem seed kernel extract	None	Poly(ε-caprolactone) Mw 50,000
REF/80/CA	None	None	Poly(ε-caprolactone) Mw 80,000
DFNSK/80/CA	De-fatted neem seed kernel extract	None	Poly(ε-caprolactone) Mw 80,000

(A)






(B)



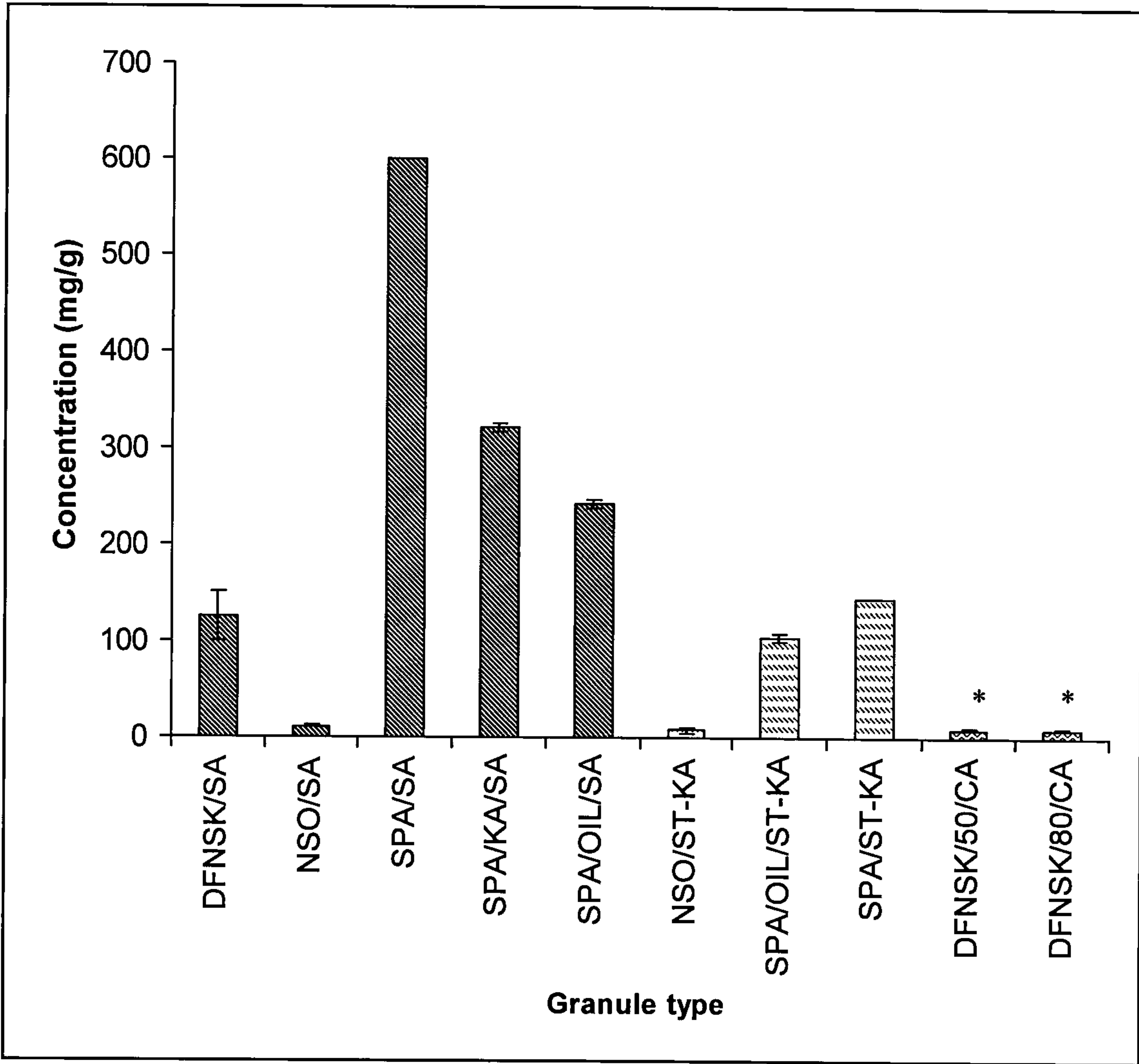
**Figure 3.5.7. Concentration of azadirachtin and related limonoids within granules.** In replicates of three, azadirachtin and related limonoids were extracted from batches of granules into methanol. The limonoids within methanol extracts were partitioned into dichloromethane and subjected to the vanillin assay for quantification. Extraction of limonoids from poly( $\epsilon$ -caprolactone) granules was not possible.

\* Quantification of poly( $\epsilon$ -caprolactone) formulations was estimated based on a granule loading of de-fatted neem seed kernel extract at 5% (w/w). The code terminology used is detailed below. Vertical lines represent  $\pm$  S.D.

-  Sodium alginate granules
-  Starch-kaolin granules
-  Poly( $\epsilon$ -caprolactone) granules

Code	Neem material	Additive	Matrix
DFNSK/SA	De-fatted neem seed kernel extract	None	Sodium alginate
NSO/SA	Neem seed oil	None	Sodium alginate
SPA/SA	Semi-purified azadirachtin	None	Sodium alginate
SPA/KA/SA	Semi-purified azadirachtin	Kaolin	Sodium alginate
SPA/OIL/SA	Semi-purified azadirachtin	Rapeseed oil	Sodium alginate
NSO/ST-KA	Neem seed oil	None	Starch-kaolin
SPA/OIL/ST-KA	Semi-purified azadirachtin	Rapeseed Oil	Starch-kaolin
SPA/ST-KA	Semi-purified azadirachtin	None	Starch-kaolin
DFNSK/50/CA	De-fatted neem seed kernel extract	None	Poly( $\epsilon$ -caprolactone) Mw 50,000
DFNSK/80/CA	De-fatted Neem seed kernel extract	None	Poly( $\epsilon$ -caprolactone) Mw 80,000





### 3.5.2. The release of azadirachtin and related limonoids from granular formulations

The cumulative percentage release of azadirachtin and related limonoids from the granular formulations is shown on figure 3.5.8. The various granules exhibited differences in the rate of release and in the total percentage of material liberated from the formulations. All granules showed incomplete release over the 28-day (672 hours) study period. Total percentages of material released by these formulations at 28 days, ranged from 1.5% (for poly( $\epsilon$ -caprolactone) granules containing de-fatted neem seed kernel extract) to 43% (for sodium alginate granules containing semi-purified azadirachtin and rapeseed oil). Azadirachtin and related limonoid release was confirmed by reversed-phase HPLC (figure 3.5.9).

The speed that granules released azadirachtin and related limonoids was affected by the type of granule matrix, the type of neem seed extract loaded and the presence of additive in the formulation. Generally, starch-kaolin granules released limonoids the most rapidly (12%-43% at 28 days). The addition of rapeseed oil to these formulations increased the percentage released during the initial 168 hours by 2%. However, during the following 504 hours, unmodified versions released an increased 3% compared to those formulations modified with oil. Granules based on sodium alginate released limonoids more slowly (6%-39% at 28 days). The rate of release was increased through the addition of kaolin clay and rapeseed oil by 7% and 14% respectively over the 28-day period. Poly( $\epsilon$ -caprolactone) granules released an insignificant amount of limonoids (1.5%-2.2% at 28 days) with little difference being recorded between formulations based on different molecular weight polymers.

The formulations can be ranked according to the total percentage of azadirachtin and related limonoids released after the 28-day study period: poly( $\epsilon$ -caprolactone)/50,000/de-fatted neem seed kernel extract (1.5%) < poly( $\epsilon$ -caprolactone)/80,000/de-fatted neem seed kernel extract (2.2%) < sodium alginate/de-fatted neem seed kernel extract (6%) < starch-kaolin/neem seed oil (12%) < sodium alginate/neem seed oil (20%) < sodium alginate/semi-purified neem seed extract (24%) < sodium alginate/semi-purified neem seed extract/kaolin (31%) < sodium alginate/semi-purified neem seed extract/oil (38.6%) < starch-kaolin/semi-purified neem seed extract/oil (39.1%) < starch-kaolin/semi-purified neem seed extract (43%).

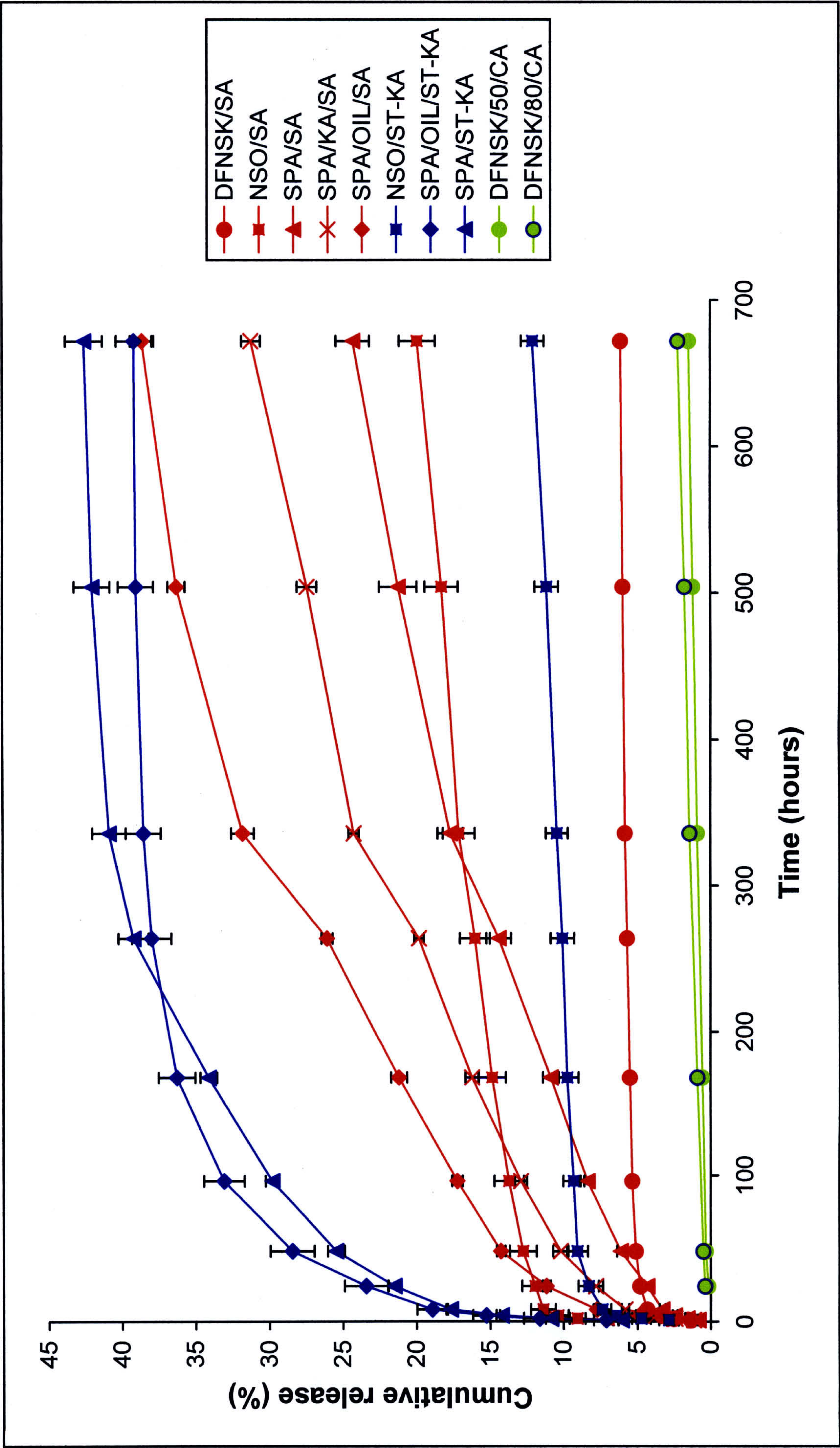
Three distinct release profiles can be identified following the addition of the granules to water. The first type was exhibited by all formulations composed of starch-kaolin, and those of sodium alginate that contained neem seed oil or de-fatted neem seed kernel extract. These were characterised by a brief, initial period, during which release occurred rapidly. This stage was followed by a prolonged period of extremely slow release. Sodium alginate granules containing semi-purified azadirachtin, with or without additives, produced the second type of release. These were distinguishable by a near-linear release rate that was sustained over the study period. Finally, the third release profile was exhibited by poly( $\epsilon$ -caprolactone) formulations. In this instance, release occurred at an extremely slow rate during the 28-day time course.



Figure 3.5.8. **The release of azadirachtin and related limonoids from granular formulations.** Batches of granules, in replicates of four, were placed into separate flasks containing distilled water and sodium azide. These were maintained in the dark on an oscillating shaker at 22°C. Release was recorded at intervals over a 28-day period. Quantification was achieved by subjecting aliquots of water containing limonoids to the vanillin assay. The code terminology used is detailed below. Vertical lines represent  $\pm$  S.D.

Code	Neem material	Additive	Matrix
DFNSK/SA	De-fatted neem seed kernel extract	None	Sodium alginate
NSO/SA	Neem seed oil	None	Sodium alginate
SPA/SA	Semi-purified azadirachtin	None	Sodium alginate
SPA/KA/SA	Semi-purified azadirachtin	Kaolin	Sodium alginate
SPA/OIL/SA	Semi-purified azadirachtin	Rapeseed oil	Sodium alginate
NSO/ST-KA	Neem seed oil	None	Starch-kaolin
SPA/OIL/ST-KA	Semi-purified azadirachtin	Rapeseed oil	Starch-kaolin
SPA/ST-KA	Semi-purified azadirachtin	None	Starch-kaolin
DFNSK/50/CA	De-fatted neem seed kernel extract	None	Poly( $\epsilon$ -caprolactone) Mw 50,000
DFNSK/80/CA	De-fatted neem seed kernel extract	None	Poly( $\epsilon$ -caprolactone) Mw 80,000





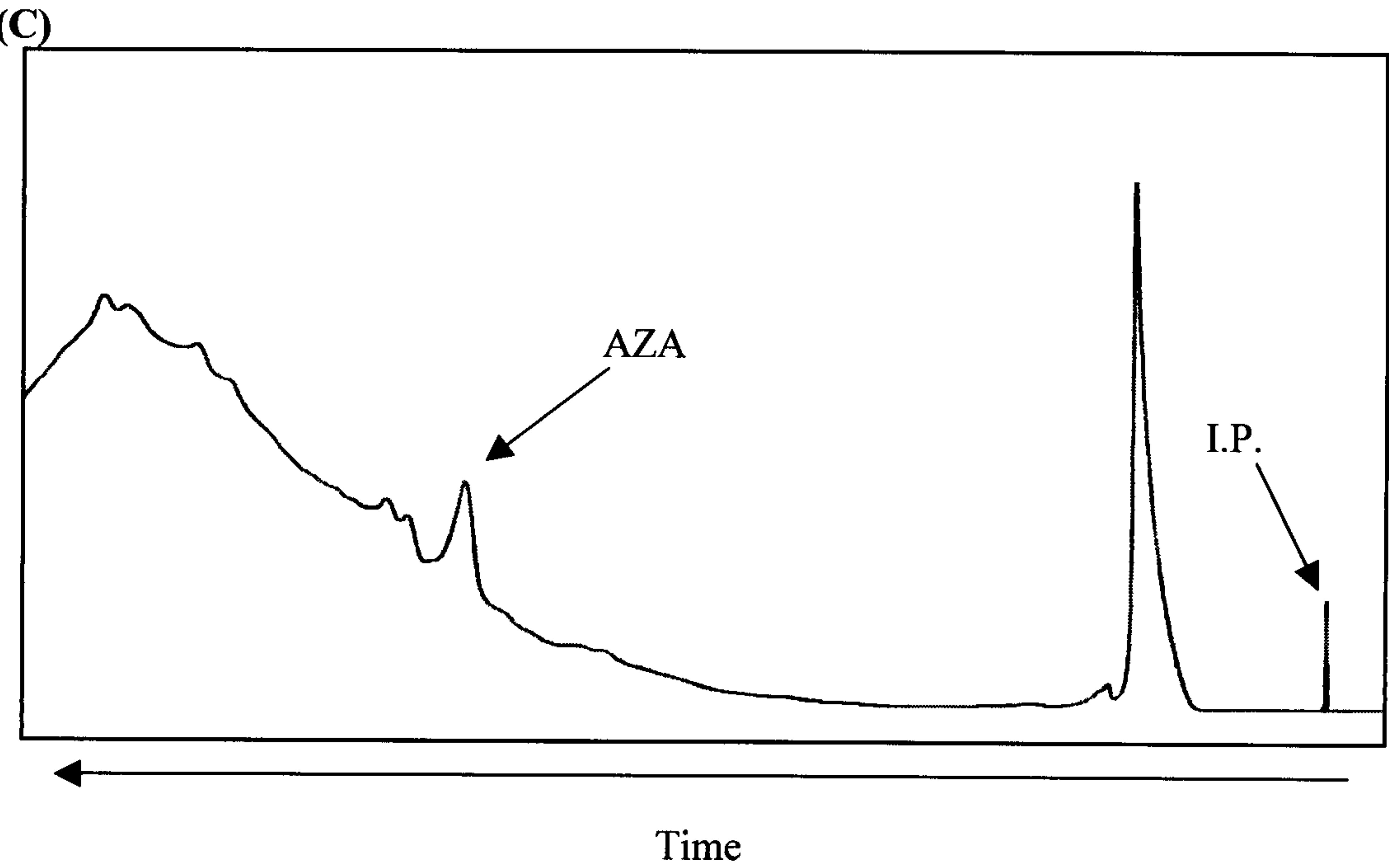
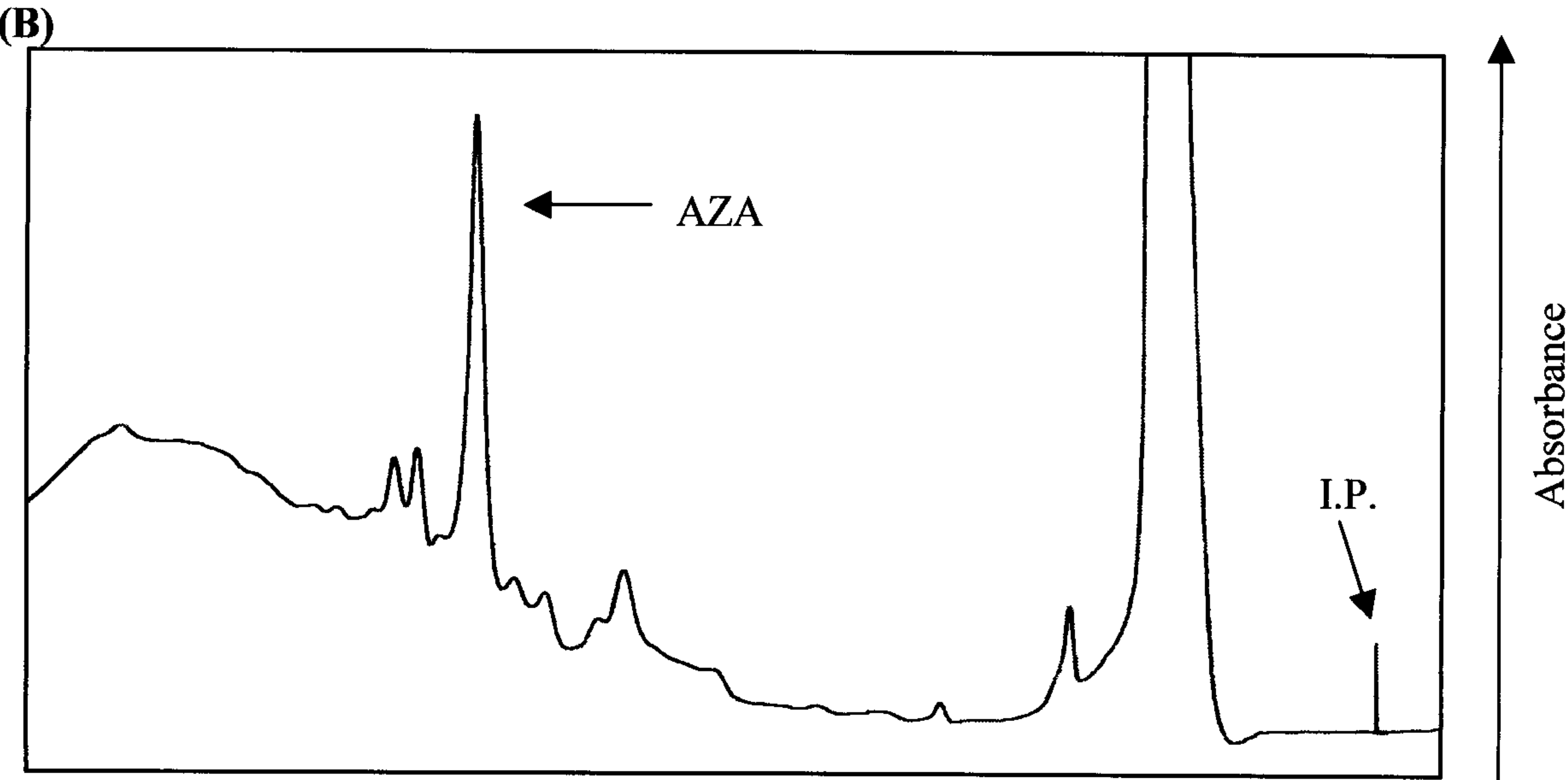
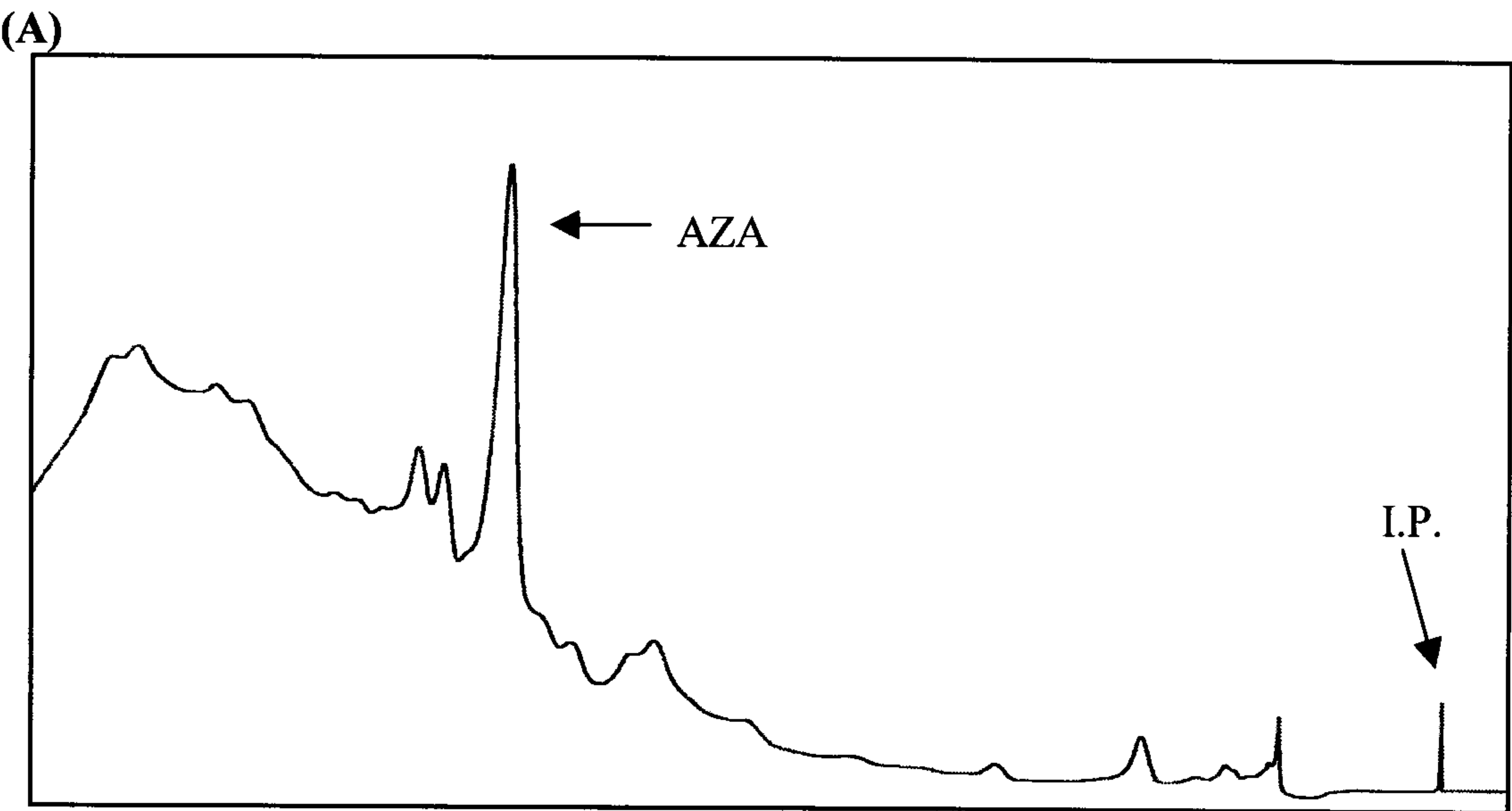


**Figure 3.5.9. Azadirachtin and related limonoids within, and released from, granular formulations.** Azadirachtin and related limonoids were extracted from granules at the beginning and end of the release-rate trial with methanol. Limonoids released by granules into water were assessed directly. Aliquots of methanol extracts or water samples were analysed using the Beckman HPLC system as described in section 3.4.10. (column = C18, solvent system = methanol/water). The ratio of methanol within the mobile phase was increased from 60% to 100% over a 10-minute period and returned from 100% to 60% over a 2-minute period. **(A)** Typical HPLC chromatogram of azadirachtin and related limonoids extracted from sodium granules prior to release study. **(B)** Typical HPLC chromatogram of azadirachtin and related limonoids released from sodium alginate granules into water. **(C)** Typical HPLC chromatogram of azadirachtin and related limonoids remaining within granules following a 28-day immersion in water. The increasing baseline absorption corresponds to the increasing concentration of methanol within the solvent system.

Key: I.P.= Injection point

AZA = Azadirachtin-A (Retention time: 11.9min)





### 3.5.3. Granule swelling study

After immersion in water, granules exhibited different swelling characteristics (figures 3.5.10. & 3.5.11.). Sodium alginate granules rapidly increased in both size and weight during the initial 24 hours within water. This rapid increase was followed by an extended period, where both granule size and weight continued to increase slowly. Starch-kaolin granules increased extremely rapidly in both size and weight during the first 8 hours after immersion in water. Following this period, granule size, but particularly weight, decreased for the remainder of the study. Poly( $\epsilon$ -caprolactone) granules were distinct from other formulations, in that both granule size and weight did not significantly change during the 28-day immersion in water.

### 3.5.4. The breakdown of azadirachtin and related limonoids within granular formulations after 28 days immersed in water

The percentage of azadirachtin and related limonoids degraded within granules, following the 28-day immersion in water, varied between 15% and 57% (figure 3.5.12.). Breakdown within sodium alginate granules was between 15% and 47%. Formulations containing either kaolin clay or rapeseed oil increased the amount of breakdown by 18% and 32% respectively when compared to unmodified versions. Degradation within starch-kaolin granules varied between 24% and 57% and was increased through the addition of rapeseed oil by 5% when compared to unmodified version of the formulation. The estimation of breakdown within poly( $\epsilon$ -caprolactone) formulations was not possible because of extreme granule hardness which prevented azadirachtin and related limonoid recovery.

3.5.5. The shelf life of sodium alginate granular formulations

The breakdown of azadirachtin and related limonoids within granules stored in glass containers is shown on figure 3.5.13. Formulations that were stored at  $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$  in the dark had a half-life of 323 days, while those kept in a position that received natural, direct sunlight had a half-life of 251 days.



Figure 3.5.10. **Granule (weight) swelling study.** Batches of granules, in replicates of four, were placed into separate flasks containing distilled water and sodium azide. These were maintained in the dark on an oscillating shaker at 22°C. Single granules in replicates of five were removed from each flask at intervals over a 28-day period. These were rolled on aluminium foil to remove surface water and weights were recorded. **(A)** Typical change in sodium alginate granule weight. **(B)** Typical change in starch-kaolin granule weight. **(C)** Typical change in poly( $\epsilon$ -caprolactone) granule weight. Vertical lines represent  $\pm$  S.D.

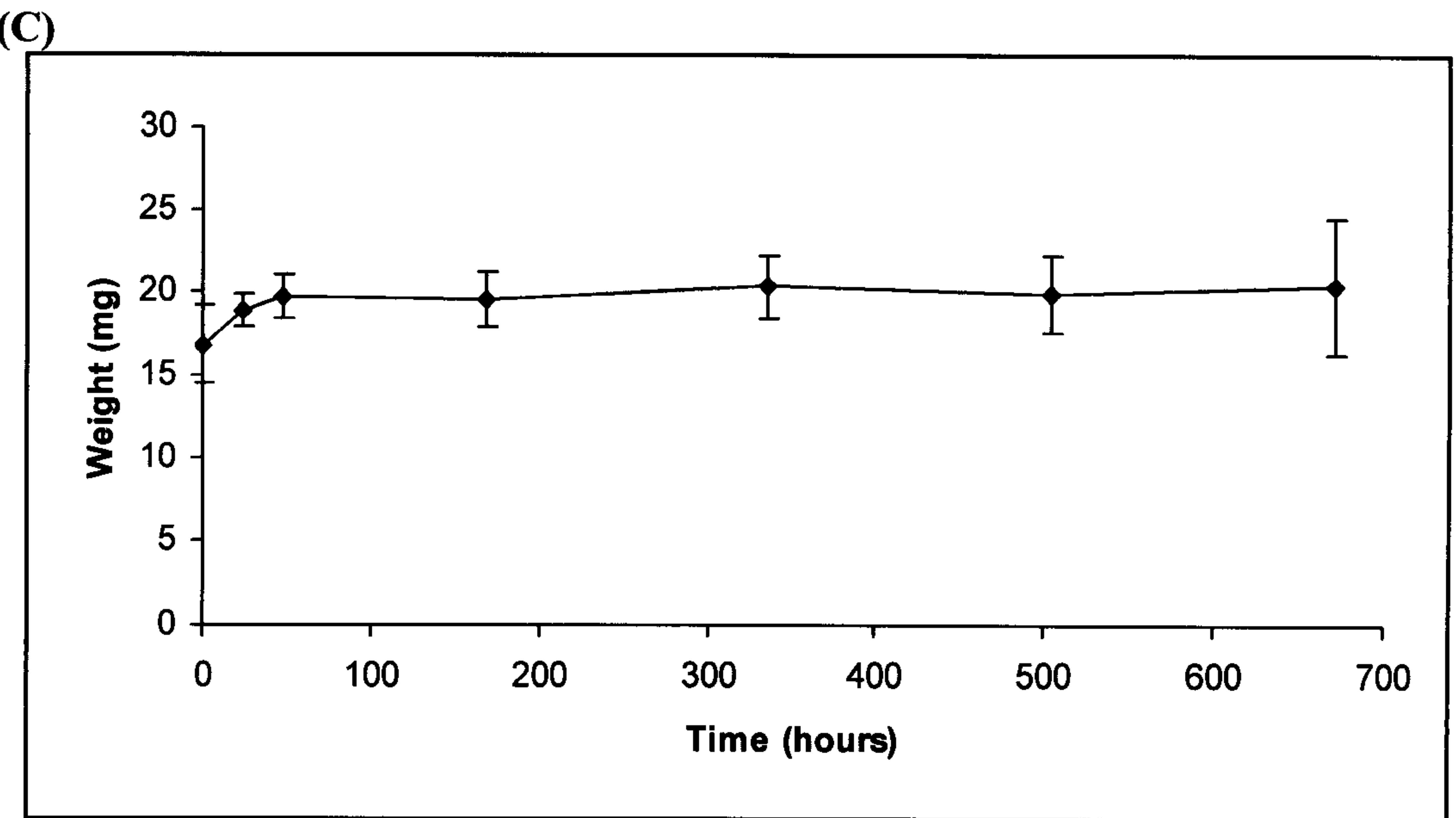
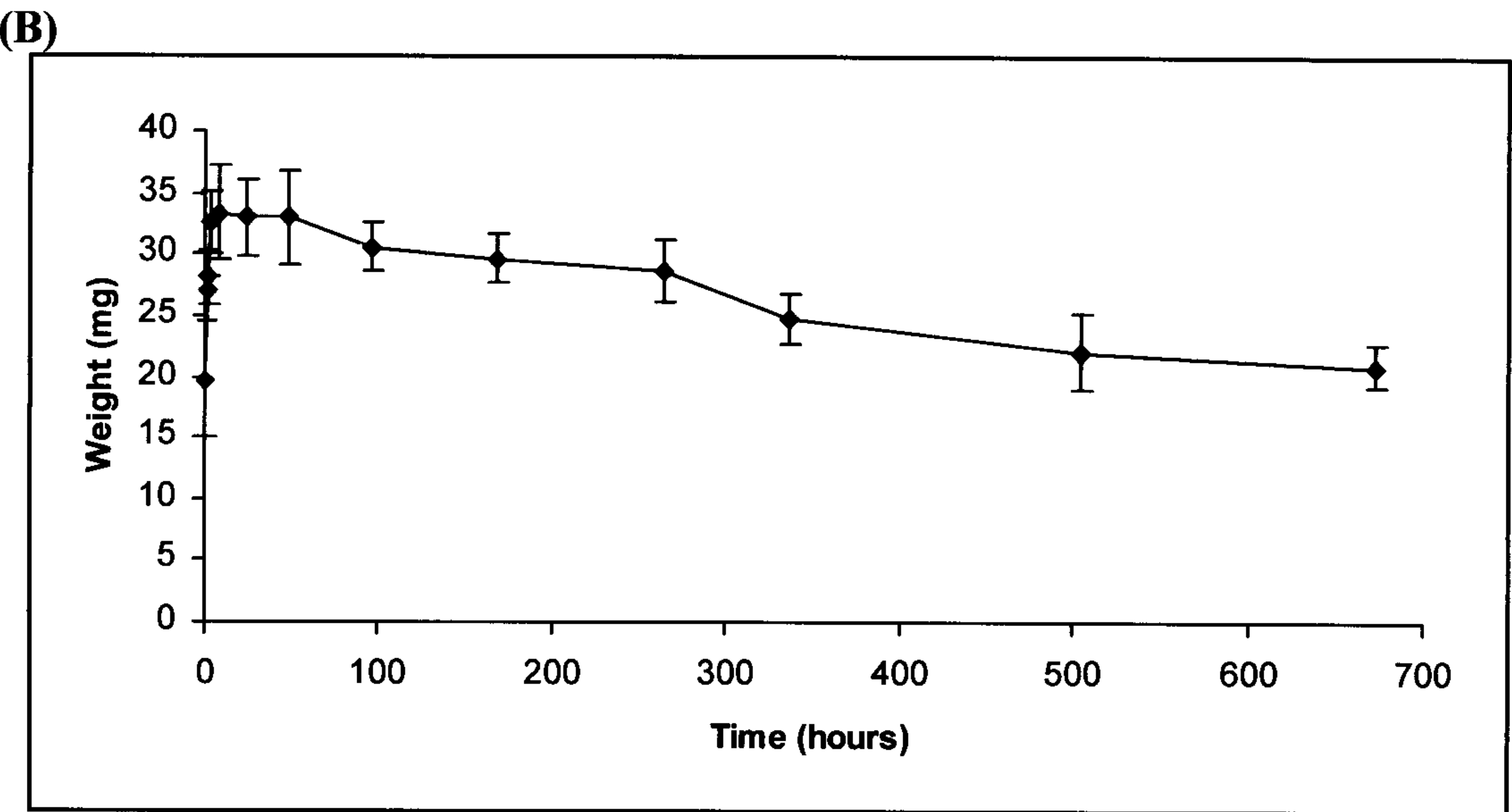
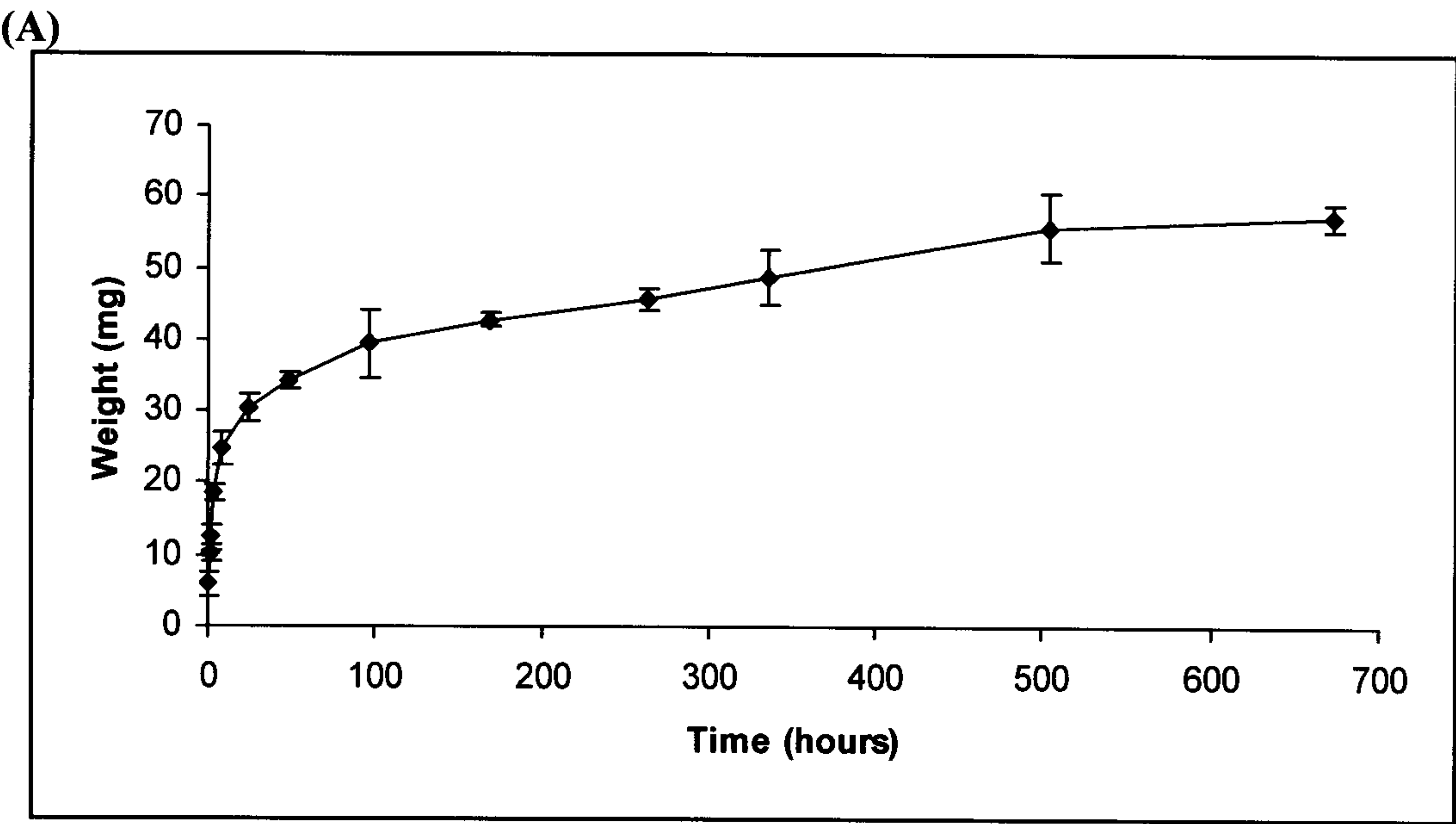
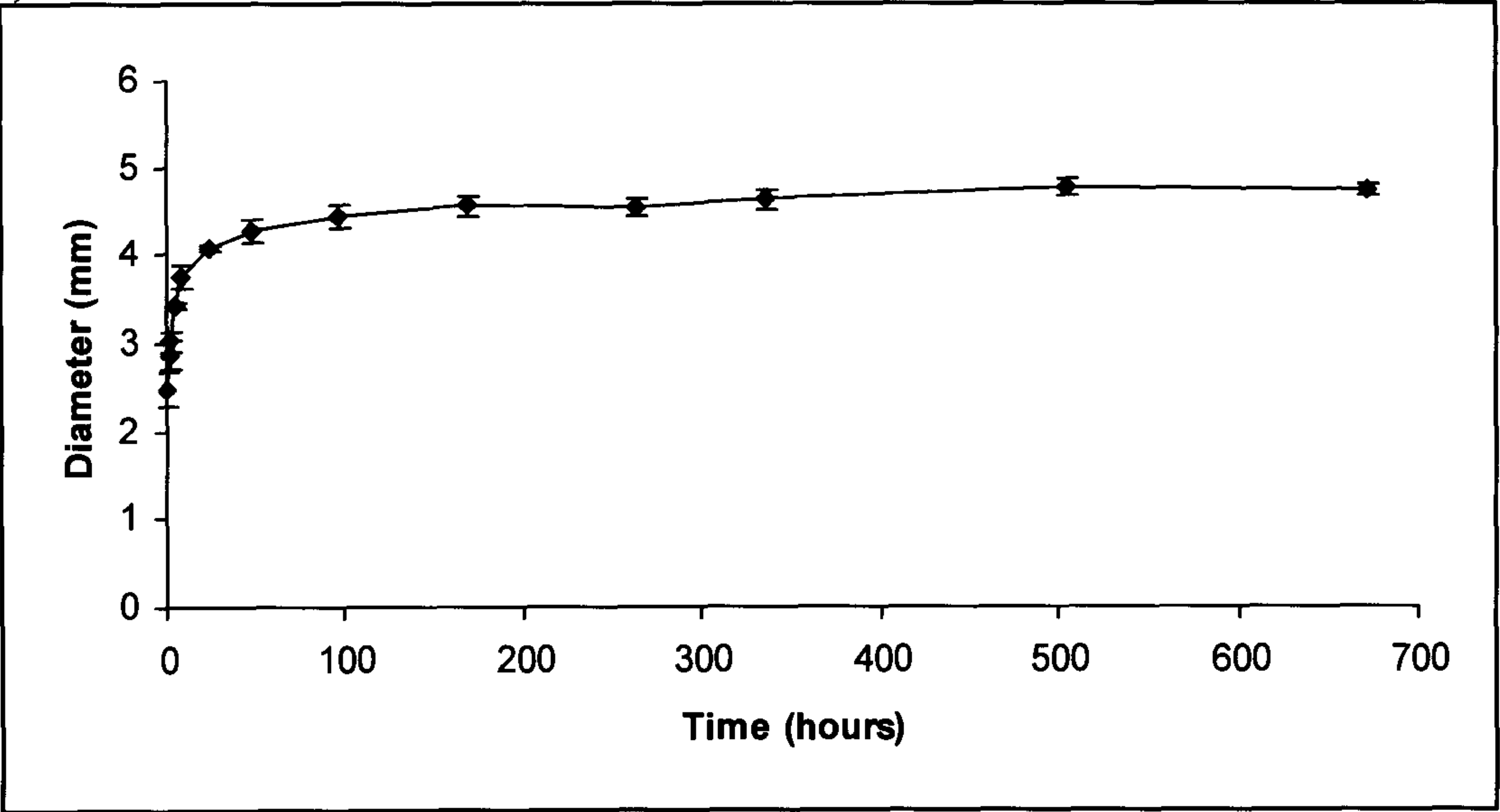


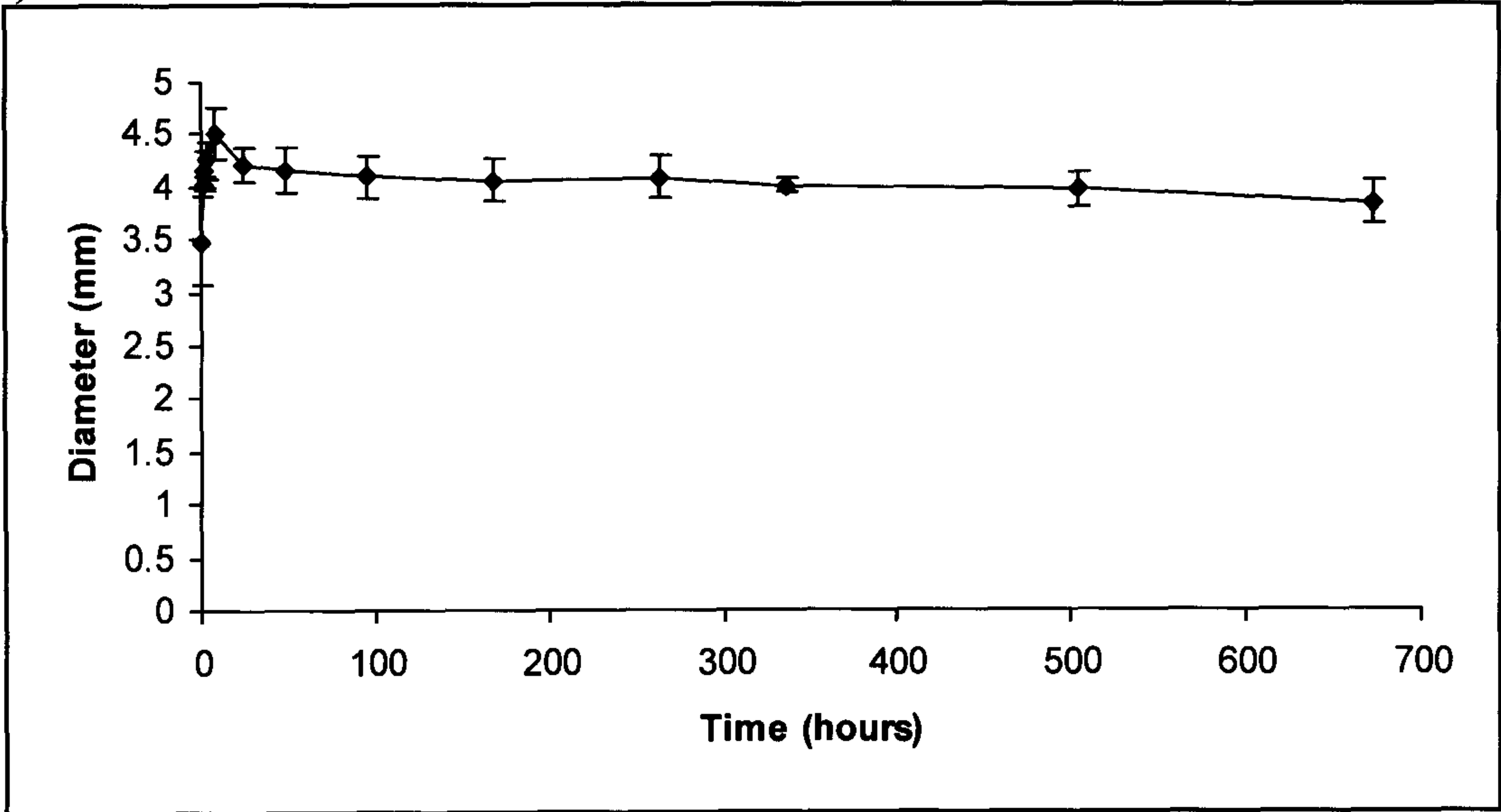
Figure 3.5.11. **Granule (diameter) swelling study.** Batches of granules, in replicates of four, were placed into separate flasks containing distilled water and sodium azide. These were maintained in the dark on an oscillating shaker at 22°C. Single granules in replicates of five were removed from each flask at intervals over a 28-day period. These were rolled on aluminium foil to remove surface water and diameters were recorded. **(A)** Typical change in sodium alginate granule diameter. **(B)** Typical change in starch-kaolin granule diameter. **(C)** Typical change in poly( $\epsilon$ -caprolactone) granule diameter. Vertical lines represent  $\pm$  S.D.



(A)



(B)



(C)

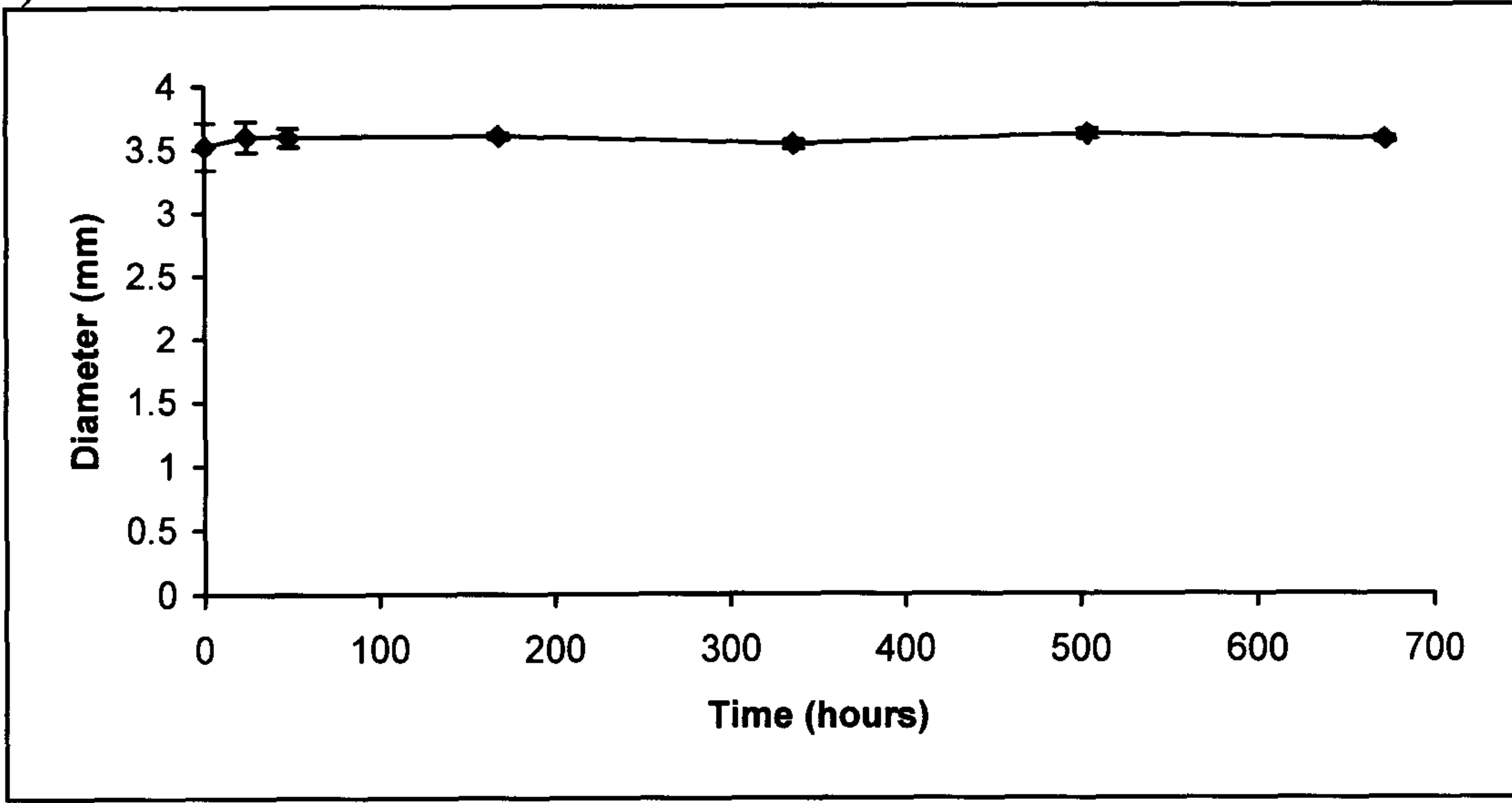




Figure 3.5.12. The breakdown of azadirachtin and related limonoids within granular formulations after 28 days immersed in water. Batches of granules, in replicates of four, were placed into separate flasks containing distilled water and sodium azide. These were maintained in the dark on an oscillating shaker at 22°C for 28 days. During this period the release of limonoids was quantified. After the 28 days, granules were removed from each flask and allowed to air-dry. Batches of granules in replicates of three were taken and azadirachtin and related limonoids extracted into methanol. The limonoids within methanol extracts were partitioned into dichloromethane and subjected to the vanillin assay for quantification. Percentage breakdown was estimated by comparing the percentage of azadirachtin and related limonoids released from granules to the percentage of azadirachtin and related limonoids remaining within granules. Unaccounted material was assumed to have degraded. Estimation of limonoid breakdown within poly( $\epsilon$ -caprolactone) formulations was not possible due to extreme granule hardness which prevented limonoid recovery. The code terminology used is detailed below. Vertical lines represent  $\pm$  S.D.

-  Sodium alginate granules
-  Starch-kaolin granules

Code	Neem material	Additive	Matrix
DFNSK/SA	De-fatted neem seed kernel extract	None	Sodium alginate
NSO/SA	Neem seed oil	None	Sodium alginate
SPA/SA	Semi-purified azadirachtin	None	Sodium alginate
SPA/KA/SA	Semi-purified azadirachtin	Kaolin	Sodium alginate
SPA/OIL/SA	Semi-purified azadirachtin	Rapeseed oil	Sodium alginate
NSO/ST-KA	Neem seed oil	None	Starch-kaolin
SPA/OIL/ST-KA	Semi-purified azadirachtin	Rapeseed oil	Starch-kaolin
SPA/ST-KA	Semi-purified azadirachtin	None	Starch-kaolin

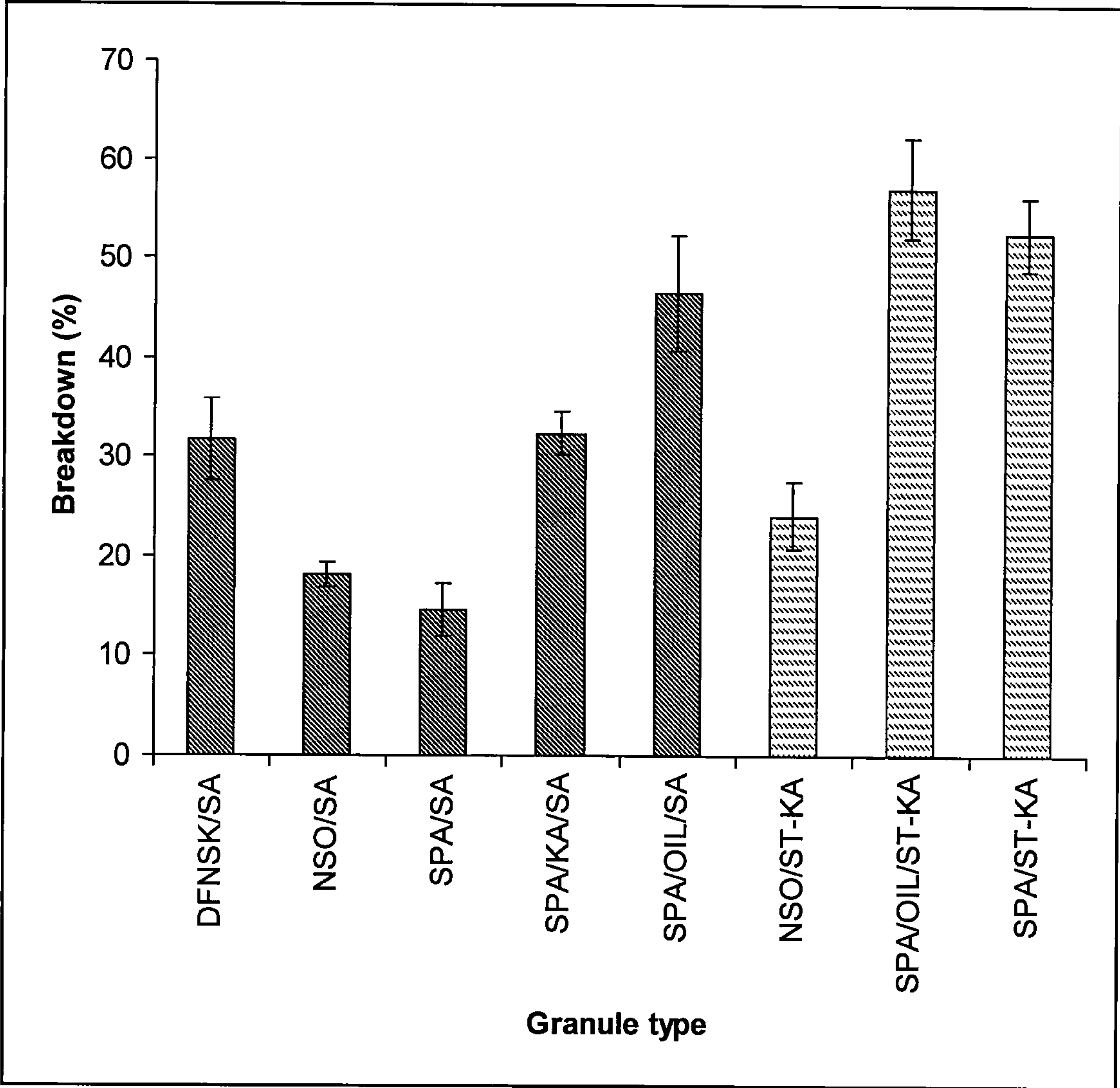
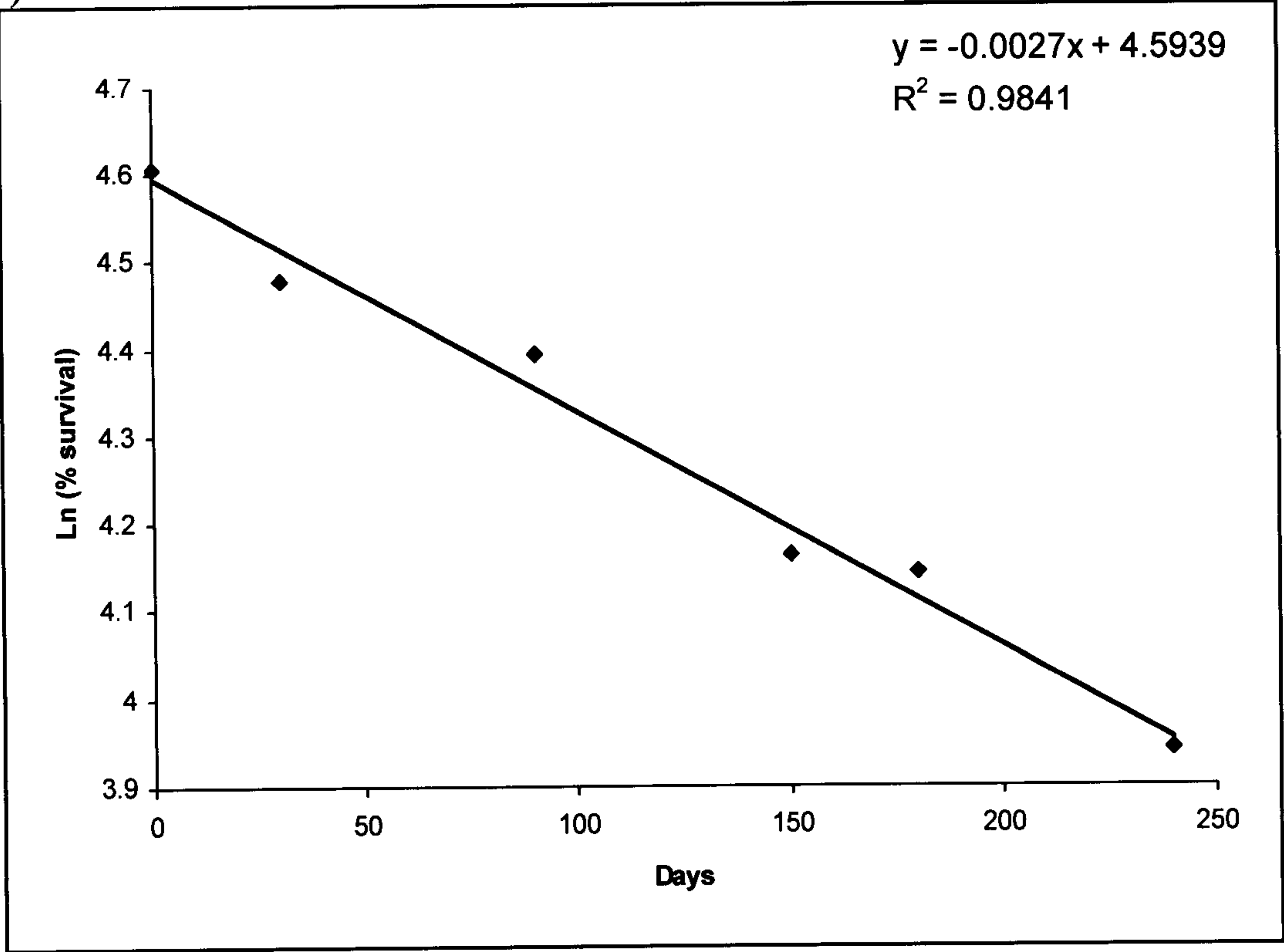


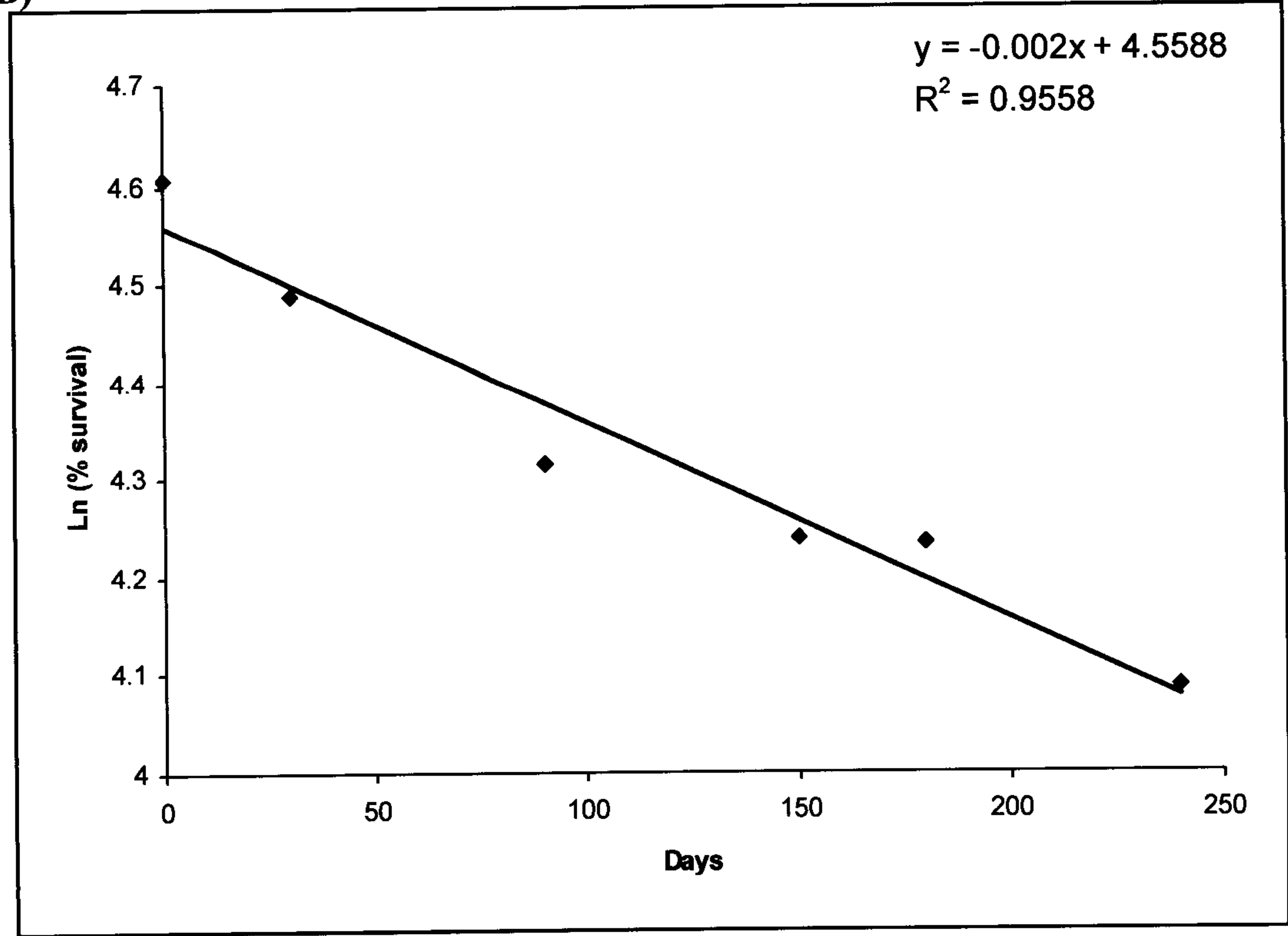


Figure 3.5.13. **The shelf life of sodium alginate granular formulations.** Batches of sodium alginate granules containing semi-purified azadirachtin were placed into separate sealed glass containers. Separate batches in replicates of two were stored at 22°C in the dark or in a position exposed to natural, direct sunlight. Quantities of granules, in replicates of three, were removed from each batch at intervals during an 8-month period and azadirachtin and related limonoids extracted into methanol. Limonoids within methanol extracts were partitioned into dichloromethane and subjected to the vanillin assay for quantification. Half-life was calculated from plots of natural log (percentage of material remaining) against time. **(A)** Breakdown of azadirachtin and related limonoids within granules stored in natural, direct sunlight. **(B)** Breakdown of azadirachtin and related limonoids within granules stored in the dark.

(A)



(B)



### **3.6. Discussion**

#### **3.6.1. Granule production**

Many procedures have been evaluated for the preparation of controlled-release granular formulations (Johnson & Pepperman, 1996). Most of this research has been conducted to improve the application efficiency of herbicides to the soil rather than insecticides. There have been however, relatively few attempts to develop granular neem based formulations that exhibit improved application characteristics when compared to conventional formulations. Nevertheless, the research presented within this chapter has produced a number of new formulations that can be considered for this purpose.

The granular formulations developed varied in size, weight and colour (figure 3.5.1. to figure 3.5.6.). These properties were dependent on the granule matrix type (e.g. sodium alginate, starch-kaolin or poly( $\epsilon$ -caprolactone)), the neem extract loaded (e.g. neem seed oil, de-fatted neem seed kernel extract or semi-purified azadirachtin) and whether an additive (e.g. kaolin-clay or rapeseed oil) was included into the formulation. Because dried granule diameter ranged from 1.9mm-3.6mm, they are generally larger than the typical granule size range of 0.2mm-2.36mm (Matthews, 2000). However, granules (e.g. clay-filled alginate and carboxymethylcellulose) developed by Connick *et al.*, (1982), were similar in terms of size to many of the formulations produced. There is little information in the literature regarding typical or ideal granule weight, although this should be sufficient to permit the penetration of crop foliage during applications to soil or water bodies (Matthews, 2000). However,



in comparison with experimental work conducted by Connick (1982), the granules produced from this work were significantly heavier.

### 3.6.2. Granule release

The release of azadirachtin and related limonoids from granules was assessed using the vanillin assay. As discussed in Chapter 2, this method offered the possibility of a rapid and specific procedure, which could be used to quantify azadirachtin within crude neem extracts. However, the assay was shown not to be specific for azadirachtin and reacted to a greater or lesser extent with all neem limonoids tested (e.g. salannin and nimbin). Therefore, it was suggested that the procedure might be more appropriate for giving a relative estimate on the total amount of azadirachtin and its related limonoids present within samples. Consequently, as the release studies required that a considerable number of samples be analysed, the assay was deemed suitable for rapidly estimating the release of azadirachtin and related limonoids from granules into water.

Granule formulations were prepared by heterogeneously dispersing the active ingredient throughout a polymeric matrix. This matrix often takes the form of a honeycomb structure that collapses upon drying but swells upon rewetting (Mogul *et al.*, 1996). Consequently, the release of material from formulations based on these matrices generally involves the simultaneous absorption of water and desorption of pesticide via a swelling controlled diffusion mechanism (Lee, 1985). This mechanism would appear to have been exhibited by all formulations (except for those based on

poly( $\epsilon$ -caprolactone)), where increases in granule size and weight were accompanied by the release of azadirachtin and related limonoids.

Poly( $\epsilon$ -caprolactone) granules released azadirachtin and related limonoids very slowly (figure 3.5.8.). It is likely that extreme formulation hardness and low water-solubility of the polymer prevented the required movement of water into the formulation to induce release. This statement would appear to be supported by the observation that these granules were unchanged in terms of size or weight, following their immersion in water. Nevertheless, these biodegradable plastics have been successfully used in the controlled-release of pesticides (Solvay, 1998). However, in these situations it is likely that release has followed microbial breakdown or physical erosion and deterioration of polymer matrices (Sivalingam & Madras, 2003 & Yavuz *et al.*, 2002). Consequently, the addition of sodium azide into incubating waters, which would have prevented microbial action on not only neem limonoids but also on the polymer, may have additionally contributed to the failure of these formulations to release. Therefore, the methodology used during the studies may not have reflected realistic release conditions.

Starch-kaolin granules were in many respects the opposite of poly( $\epsilon$ -caprolactone) formulations in that these were fragile, rapidly releasing materials. The speed of release can be attributed to the observation that these formulations rapidly absorbed water and then began to disintegrate. This disintegration of the matrix is reflected by the reduction in both weight and size of the granule over time (figures 3.5.10.B. and 3.5.11.B.) This physical breakdown of the solid carrier used in a granule formulation has been linked to increased release rates (Kenawy, 1998). In addition, starch-kaolin

granules had surface cavities (figure 3.5.3), which would have improved the penetration of water into the formulation as well as increased granule surface area. Both of these properties have also been linked to increased release rates (Cotterill *et al.*, 1996).

The plateau in release profiles from starch-kaolin granules loaded with semi-purified azadirachtin, with or without rapeseed oil are unexpected results, in that these would suggest that release prematurely stops (figure 3.5.8.). Pepperman & Kuan (1995) recorded a similar effect when studying the release of alachlor from controlled-release formulations. A possible explanation for this was given, whereby after an initial rapid release, an equilibrium can be reached between the pesticide within the granule and that within the water surrounding the granule. However, in the trials presented within this chapter, water was continually replaced (at each assessment point), therefore an equilibrium being established seems unlikely. Consequently, to explain this effect, the percentage released must be related to the percentage degraded within granules. Azadirachtin and related limonoids were significantly (up to 57%) degraded within starch-kaolin matrices during the 28-day immersion in water (figure 3.5.12.). Therefore, it is more likely that release was offset by breakdown and it is this loss that is responsible for slowing and prematurely completing release. The likely reason for starch-kaolin granules causing a high percentage breakdown of neem limonoids was that 8 molar sodium hydroxide was required to gel starch slurries. This resulted in the gel having a pH of 13, under which, azadirachtin has been reported as highly unstable (Jarvis *et al.*, 1998). This effect of pH on the stability of azadirachtin was confirmed at a later point in the project (see chapter 4 figure 4.5.4.).



Sodium alginate granules were intermediate in formulation strength and in contrast to starch-kaolin granules, these formulations retained structural integrity throughout the 28-day immersion in water, with no visible signs of matrix breakdown. Following their addition to water, these granules released azadirachtin and related limonoids in a manner that mirrored granule swelling (figures 3.5.8; 3.5.10.A. and 3.5.11.A.). This release has been described as characteristic of diffusion-controlled matrix systems, which generally exhibit a release behaviour starting with a substantial release that gradually diminishes with time (Lee, 1985).

The release mechanism of alginate granules has been described as having significant capacity of being modified. In this research, this was attempted through the addition of clay or oil to formulations. Both additives have been successfully used to increase the duration of herbicide release from granular formulations (Pepperman & Kuan, 1995). However, when these materials were incorporated into formulations containing azadirachtin and related limonoids, the speed of release was unexpectedly increased. There are a number of possible reasons as to why this occurred.

Clays, such as kaolin have been shown to slow release when incorporated into granular formulations (Johnson & Pepperman, 1998). This effect is dependant on the enhanced adsorption of pesticide to the clay within the granule matrix (Nennemann *et al.*, 2001). However, it is possible that azadirachtin and related limonoids are not strongly absorbed by the clay additive (this suggestion was supported by studies conducted later in the project, see chapter 4 figure 4.5.5.) and therefore, release would not be slowed in comparison to unmodified versions. Fleming *et al.*, (1992) recorded a similar effect during the study of atrazine movement from controlled-release

formulations based on starch. Therefore, a possible reason for the increased release rates from these formulations was that the overall absorptive capacity of the alginate granule was reduced through the presence of clay within the matrix.

The addition of oils to granular formulations can reduce release rates by altering the physical properties of the carrier employed (e.g. linseed oil on the surface of granules polymerises on drying to form a coating with increased density) or alternatively, by producing a partitioning effect (i.e. the active ingredient is partitioned between the oil within the granule and the surrounding water) (Pepperman & Kuan, 1993 & 1995). Consequently, granules loaded with rapeseed oil may have failed to reduce release rates through a combination of two properties. The first is explained by Pepperman & Kuan (1995) who noted that sunflower oil (e.g. rapeseed) containing granules did not exhibit a polymeric coating when compared to formulations produced with linseed oil. It was concluded that the non-drying property of sunflower oil prevented the formation of this additional barrier to diffusion. The second likely reason may be explained by the granule structure. Dried sodium alginate granules containing rapeseed oil were soft in comparison to unmodified versions and expelled considerable amounts of oil on compression. Therefore, under dynamic incubation conditions, small amounts of oil, containing large quantities of azadirachtin may have been released. This suggestion is supported by observations made in chapter 4 (figure 4.5.3.) where azadirachtin was shown to partition approximately 10 times more favourably into a lipid phase than a water phase.

### 3.6.3. Formulation stability

The stability of azadirachtin and related limonoids was assessed within granules that had been immersed in water for 28 days (figure 3.5.12). This indicated that the addition of either rapeseed oil or kaolin clay to formulations increased breakdown. Pepperman & Kuan (1992) noted that the addition of linseed oil to granular, alginate formulations containing metribuzin, caused degradation of the herbicide. The percentage loading of linseed oil was positively correlated to percentage breakdown in this instance; however, the cause of this effect was not identified. To date there has been no published research that has recorded kaolin clay promoting active ingredient breakdown. Therefore, the cause of this effect remains unexplained.

Sodium alginate granules loaded with semi-purified azadirachtin were chosen to evaluate the shelf life stability of formulations (figure 3.5.13.). This work indicated that storing granules in the dark increased the half-life of azadirachtin and related limonoids within formulations when compared to granules kept in direct sunlight. However, the half-life of the limonoids was below 1 year and this may have considerable drawbacks for a potential commercial production.



## Chapter 4

### The Movement of Azadirachtin within Soil and Plant Systems

## **4.1. Introduction**

### **4.1.1. The movement of pesticides within soil**

Soil-applied pesticides require local transport systems to reach sites of uptake (i.e. roots) on target plants (Barlow, 1985). Within the soil, pesticides are transported to roots mainly by the physical process of molecular diffusion and bulk transfer with water, which moves to the root in response to hydrostatic pressure gradients that result from transpiration by the plant (Hartley & Graham-Bryce, 1980b; Sharom *et al.*, 1981 and Taiz & Zeiger, 1998).

This process is influenced by the physical/chemical properties of the pesticide concerned (e.g. volatility, water and lipid solubility, resistance to chemical change and presence of surfactants within formulations), the characteristics of the soil environment (e.g. soil texture, structure, acidity, organic carbon and mineral content as well as microbial activity) and prevailing weather conditions (e.g. temperature, ultraviolet light and rainfall) (Berglöff *et al.*, 2002; Dai *et al.*, 2001a; Gawlik *et al.*, 1997; Gong *et al.*, 2001; Hopkins, 1999; Johnson *et al.*, 1999; Ma *et al.*, 2000; Novak *et al.*, 2001 and Roy *et al.*, 2000).

However, the most important property governing the physico-chemical behaviour of pesticides in porous media, such as soils, is the way in which the chemical partitions between organic matter (e.g. partially decomposed organic residues and humic substances), mineral components (e.g. silicate clays, oxyhydroxides and amorphous materials of iron and aluminium), liquid (i.e. water containing organic and inorganic

solutes), and gaseous (e.g. nitrogen, oxygen and carbon dioxide) phases (Ding *et al.*, 2002; Hartley & Graham-Bryce, 1980a; Hesterberg, 1998 and White 1979).

#### 4.1.2. Sorption and desorption of pesticides to soil

Soil has been described as a dual sorbent system, in which organic matter functions as a partitioning medium and mineral components as conventional adsorbents which reversibly bind the polar functional groups of a pesticide through H-bonding and van der Waals bonding. In addition, ionic bonding may also be involved in the sorption of charged pesticides to the mineral fraction of the soil (Celis & Koskinen, 1999; Li *et al.*, 2003 and Spark & Swift, 2002).

A pesticide that is associated with the solid phase of a soil may be sorbed onto surfaces, or alternatively may have penetrated into amorphous interiors in a time-dependant process which is diffusion-limited (Hartley & Graham-Bryce, 1980a; Lesan & Bhandari, 2003 and Oi, 1999). Regardless of the precise sorption site, chemicals associated with the solid phase are generally immobile and are prevented from participating in solution-based reactions (Gerstl, 2000). Consequently, for most pesticides, it is this distribution (i.e. between solid and liquid phases) that determines not only mobility but also microbial breakdown and availability for uptake by plants (Celis & Koskinen, 1999; Cheah *et al.*, 1997; Gawlik *et al.*, 1999 and Wang *et al.*, 1999).



### 4.1.3. Soil sorption coefficients

Numerous physico-chemical properties have been used to characterise the adsorption of pesticides to soils. In particular, the *n*-octanol/water partition coefficient, soil sorption coefficient and organic carbon sorption coefficient have been used for this purpose (Felding, 1997; Gramatica & Guardo, 2002 and Müller, 1997).

The *n*-octanol/water partition coefficient ( $P_{ow}$ ) is used to describe the hydrophobicity of a compound. This coefficient has proved useful in predicting the adsorption of pesticides to soil, and is defined as the equilibrium concentration ( $C_e$ ) of a dissolved substance in a system that consists of two largely immiscible solvents (e.g. *n*-octanol and water) (Ellgehausen *et al.*, 1981 and Nemeth-Konda *et al.*, 2002).

$$P_{ow} = C_e (\text{octanol})/C_e (\text{water})$$

The extent and strength of pesticide sorption to soils and other geosorbent surfaces at the water/solid interface is generally expressed by the sorption coefficient,  $K_d$ , in the following linear relationship:

$$K_d = (x/m_s)/C_e$$

Where  $x/m_s$  is the concentration of pesticide in the solid phase and  $C_e$  is the pesticide solution concentration at equilibrium. Although some compounds exhibit non-linear sorption isotherms with soil, generally sorption of pesticides can be described using this linear model (Gerstl, 2000 and Wauchope *et al.*, 2002).

The majority of pesticides are organic compounds which are not ionised under the prevailing pH conditions of agricultural soils. Therefore, if the adsorption characteristics of such compounds are studied in a range of soils, a good correlation is often obtained between measured adsorption coefficients and the organic matter content of the soil (Gerstl, 2000 and Nicholls, 1988). It is often convenient therefore, to normalise the sorption coefficient to the soil organic carbon content (Martins & Mermoud, 1998). This is achieved by the soil organic carbon sorption coefficient ( $K_{oc}$ ):

$$K_{oc} = K_d/f_{oc} \text{ or alternatively } K_{oc} = (x/m_c)/C_e$$

Where  $f_{oc}$  is the mass fraction of organic carbon within the soil,  $x/m_c$  is the concentration of the pesticide in the soil organic carbon and  $C_e$  is the pesticide solution concentration at equilibrium. The  $K_{oc}$  is regarded as a “universal” parameter related to the hydrophobicity of the pesticide molecule, which is applied to a given pesticide in all soils (Wauchope *et al.*, 2002).

#### 4.1.4. The movement of pesticides within plants

Many pesticides are applied to the soil for absorption by plant roots and subsequent systemic action (Crafts & Yamaguchi, 1960; Raveton *et al.*, 1997; Singh, 1989 and Wieneke & Steffens, 1976). This form of application takes advantage of the root structure, which is better adapted for the exchange of dissolved substances than that of the leaf, which is adapted for gas exchange and is designed to avoid contact with

liquid water, through the presence of epicuticular wax deposits (Chowdhury *et al.*, 2001; Hartley & Graham-Bryce, 1980b; Kirkwood, 1999 and Stevens & Baker, 1987). Plant roots passively adsorb pesticides from the soil via either the liquid or vapour phase. The proportion taken-up by each route is governed by the physico-chemical properties of the compound, prevailing weather conditions (particularly water availability) and soil sorption kinetics (Bromilow & Chamberlain, 1995; Cayley & Hide, 1980 and Dejonckheere *et al.*, 1983).

The permeability of roots to water varies widely with age, physiological condition and water status of the plant (Hopkins, 1999). Nevertheless, the areas of the root absorbing water and solutes most efficiently are generally located a few mm to cm behind the growing tip in the apical region of the root (Hartley & Graham-Bryce, 1980b and Taiz & Zeiger, 1998). More mature regions of the root are generally less permeable to water because of the presence of a hydrophobic exodermis. However, for pesticides that are suitably lipophilic, uptake can remain significant within these regions (Sicbaldi *et al.*, 1997 and Taiz & Zeiger, 1998).

Once the root has taken up a solute from the soil solution, it must traverse the cortex in order to reach the xylem elements within the central stele, where distribution to other plant parts can occur (Hopkins, 1999). This radial transfer occurs simultaneously through two pathways. Firstly, through the apoplast pathway, whereby water moves exclusively through the cell wall space of the epidermis and cortex without crossing any membranes. In certain species, transport via this route can be significantly retarded by the presence of the Casparian strip. This strip passively retains pesticides through the presence of hydrophobic suberin (a wax-like substance



that is a modification of lignin). However, a compound that has been sorbed will be gradually eluted through this band of radial cell walls as more water passes through the root. (Edgington, 1981 and Taiz & Zeiger, 1998). Alternatively, a pesticide that is taken up by the root can travel via the symplast route, whereby water crosses cell membranes of root hairs, epidermis or cortex and moves to stele by plasmodesmata (an open channel in the cell wall containing strands of cytoplasm) and/or by membrane permeation (Campbell, 1993 and Sicbaldi *et al.*, 1997).

The extent to which a pesticide moves within each of these systems is governed primarily by the lipophilicity of the molecule. Compounds that are highly lipophilic readily cross the plasma membrane of root hairs, cortex and stele cells, and consequently, movement is primarily symplastic. In contrast, a chemical of low lipophilicity is partitioned less into lipid cell structures and therefore, movement will occur predominantly by water mass flow through the root apoplast (Sicbaldi *et al.*, 1997).

#### 4.1.5. Movement within the vascular systems of plants

Access to, and movement within the vascular system is essential for long distance transport within plants and occurs within the xylem and/or phloem vessels (Inoue *et al.*, 1998 and Kleier, 1994).

The xylem consists primarily of dead, end-perforated vessel elements and tracheids and is responsible for the transport of water, dissolved minerals and other organic molecules upward through the plant (Hopkins, 1999 and Richardson, 1975). Within

the system, water (carrying dissolved pesticide) moves in a bulk flow of sap caused by a hydrostatic pressure difference between the roots and the leaves. Negative water pressure develops in the leaf xylem as water is lost through transpiration and flow is generated by cohesion of water molecules coupled with a positive root pressure (Devine & Hall, 1990 and Hartley & Graham-Bryce, 1980b).

Pesticides that are primarily transported within the xylem are described as exhibiting an apoplastic pattern of movement. During transport, chemicals can be reversibly sorbed by vessel components such as polygalacturonic acid, cellulose and particularly lignin. The extent of this can be described by the  $P_{ow}$  value of the compound concerned (Barak *et al.*, 1983; Kirkwood, 1987 and Trapp, 2000).

In addition to transport within the xylem, non-ionised polar chemicals may also be translocated within the phloem conducting system (Bromilow *et al.*, 1987; Bromilow *et al.*, 1990b and Grayson & Kleier, 1990). The phloem consists primarily of porous, end-to-end arranged sieve elements containing living protoplasts, companion cells and phloem parenchyma. This vessel is mainly responsible for the translocation of organic materials, primarily sucrose, from sites of synthesis to storage sites or sites of metabolic demand in a process known as assimilate partitioning (Chamberlain, 1984; Hartley & Graham-Bryce, 1980b; Hopkins, 1999; Richardson, 1975 and Vaughn *et al.*, 2002).

Following passive diffusion into the sieve element, a systemic chemical will move according to an osmotically-generated pressure flow (described as a Münch-type pressure flow mechanism), which is created and maintained by loading and unloading

of photoassimilates at the source and sink tissues respectively (Chen *et al.*, 2001; Fisher, 1978 and Hopkins, 1999). Tyree *et al.*, (1979) proposed the intermediate permeability theory to explain mobility of certain pesticides within the phloem. This theory states that many compounds are capable of entering the phloem, but only those having limited permeation rates through membranes could be retained for a sufficient duration to allow long distance transport to occur. This theory was based on the assumption that compounds moving freely across membranes would rapidly equilibrate between the phloem and xylem vessels. Consequently, movement would occur predominantly in the direction of the transpiration stream (i.e. within the xylem vessel), which has a flow rate that is typically 50-100 times greater. This theory has been subsequently supported and further characterised in numerous mathematical models (Bromilow *et al.*, 1990a; Hsu *et al.*, 1988 and Kleier, 1988).

Compounds that are primarily transported within phloem vessels are said to exhibit a symplastic pattern of movement and it is this transport which has been associated with the more efficient protection of plant parts, particularly shoot apices (Chamberlain *et al.*, 1984 and Kirkwood, 1987).

Many compounds are capable of travelling within either the phloem or xylem vessels, but there are a number of pesticides that are capable of being significantly transported in both systems and these are termed “ambimobile” (Kirkwood, 1987).



#### 4.1.6. The application of azadirachtin as a soil-applied, systemic insecticide

There have been a number of studies that have investigated azadirachtin as a systemic insecticide that is absorbed by roots. These have shown that the limonoid does have considerable potential for use within this role. For example, Nisbet (1992) demonstrated that azadirachtin, absorbed by the roots of *Nicotiana clevelandii* under hydroponic conditions, could prevent adult apterous *Myzus persicae* from feeding on plants. It was concluded from this work, that the limonoid could offer a commercially viable aphicide for preventing the transmission of potato leafroll virus and potato virus Y by the aphid. Similarly, Sundaram (1996) showed that the limonoid was capable of root uptake and transport within the vascular system of young spruce trees. This author concluded that as a result of this mobility, the limonoid might have potential uses as a systemic insecticide within forestry applications. In addition, Thöming *et al.*, (2003) were able to demonstrate that when applied as a drench to the soil, azadirachtin was suitably mobile to move from application sites, through the soil and into the leaf tissue of the green bean (*Phaseolus vulgaris*). Additionally, this author was also able to show that azadirachtin solutions applied in this manner were capable of producing significant mortality within Western Flower Thrip (*Frankliniella occidentalis*) populations feeding on the leaves. In similar studies conducted by Basedow (2003), applications of crude neem seed extracts to soil were shown to be successful at causing the mortality of various species of aphid feeding on the aerial regions of *Vicia fabae* plants.

## **4.2. Aims**

As previously discussed, numerous authors have suggested that azadirachtin exhibits significant potential as a soil-applied systemic insecticide. However, there has been very limited quantitative research that has studied the physical and chemical behaviour of the limonoid in this respect. Therefore, the suitability of azadirachtin as a systemic insecticide, which is applied to the soil within a granular formulation, remains unclear.

Consequently, the aim of this chapter was to assess, in detail, the physico-chemical behaviour of azadirachtin within the soil and plant environment. In particular, the level of the limonoid's mobility and stability within these systems would be investigated. Additionally this chapter would also study the uptake of azadirachtin by plant roots from the aqueous phase of soils. These results could then be used to determine if azadirachtin is physically and chemically appropriate for use within the role proposed by this project.

## **4.3. Materials**

Nasturtium plants (Empress of India) and compost (100% peat) were purchased from B&Q. All soils were kindly supplied by Dr Hugh Flowers (University of Glasgow) and are described in figure 4.5.1. Azadirachtin-A was obtained from flash column chromatography (described in Chapter 2) and had a purity of approximately 95% as determined by HPLC.

## **4.4. Experimental**

### **4.4.1. Radiolabelling of azadirachtin**

Azadirachtin (described above) was supplied to RC Tritec Ltd. (Teufen, Switzerland), where the 22, 23 enol ether bond was hydrogenated with two tritiated hydrogen atoms to produce [22, 23,  $^3\text{H}_2$ ]-dihydroazadirachtin.

### **4.4.2. Preliminary azadirachtin water solubility study**

The preliminary investigation into azadirachtin's water solubility was conducted using the guidelines presented in Commission Directive 92/69/EEC. Azadirachtin (10mg) was added to increasing amounts of distilled water (0.01ml-10ml) at room temperature. After each addition, the mixture was shaken for 10 minutes and visually checked using a binocular microscope for undissolved parts of the sample. The volume of water required to achieve complete dissolution of the sample indicated the approximate solubility. This result was used to determine the volume of distilled water used in the final water solubility study.

### **4.4.3. Final azadirachtin water solubility study**

The final investigation into azadirachtin's water solubility was conducted using the guidelines presented in Commission Directive 92/69/EEC. Azadirachtin (50mg) was added to three separate glass vessels (15ml capacity). Distilled water (10ml) was then added to each vessel, which was sealed using a glass stopper. The closed vessels were



attached to a rotary shaker and mixed at  $30^{\circ}\text{C} \pm 1^{\circ}\text{C}$  in the dark. After 1, 5 and 7 hours, a single vessel was removed. The vessels were allowed to equilibrate in the dark, at room temperature for 1 hour with occasional shaking. Vessel contents were then centrifuged (3000rpm for 3 minutes) at room temperature. An aliquot (2ml) was taken and filtered using a syringe fitted with a  $0.2\mu\text{m}$  syringe filter. The absorbance at 217nm was recorded using a Beckman, DU 530, UV/VIS spectrophotometer equipped with a 10mm quartz cell (Hellma, QS, 1.000) and azadirachtin's solubility at each time interval quantified by the equation:

$$A = \epsilon \times c \times l$$

Where  $A$  is absorbance (AU),  $\epsilon$  is the molar extinction coefficient ( $\text{l.mole}^{-1}$ ),  $c$  is the molar concentration of the solution ( $\text{mole.l}^{-1}$ ) and  $l$  is the pathlength (cm). The precise solubility was determined by taking the mean value of the three assessment points

#### 4.4.4. Aqueous hydrolysis of [ $^3\text{H}$ ]-dihydroazadirachtin

Buffer solutions of distilled water at pH 5 (4.45mM citric acid, 10.30mM  $\text{Na}_2\text{HPO}_4$ ), pH 7 (1.77mM citric acid, 16.53mM  $\text{Na}_2\text{HPO}_4$ ) and pH 9 (5.00mM glycine, 0.88mM NaOH) that contained 0.01% sodium azide were prepared. Volumes (50ml) of these were added to separate glass jars that contained [ $^3\text{H}$ ]-dihydroazadirachtin (0.5mCi). These jars were sealed, stored in the dark at  $22^{\circ}\text{C} \pm 0.01^{\circ}\text{C}$  and analysed at regular intervals appropriate to the rate of breakdown (varying from hours to days). At assessment points, aliquots (5ml) were removed and [ $^3\text{H}$ ]-dihydroazadirachtin partitioned into dichloromethane (0.5ml) using the method described in section

4.4.19. The dichloromethane collections were evaporated using nitrogen gas. Dried extracts were taken up in methanol (200 $\mu$ l) and aliquots (50 $\mu$ l), in replicates of three, were analysed by TLC (see section 4.4.25.). Bands were visualised by exposing plates to autoradiography for 24 hours (see section 4.4.21.). Quantification was achieved using the Image J computer programme.

4.4.5. *N*-octanol/water partition coefficient of azadirachtin and [ $^3$ H]-dihydroazadirachtin

The partition of azadirachtin and [ $^3$ H]-dihydroazadirachtin between *n*-octanol and distilled water was conducted using the guidelines presented in Commission Directive 92/69/EEC. Bulk volumes of *n*-octanol and distilled water were mutually saturated by mixing with each other at room temperature for 24 hours. After mixing, both phases were allowed to separate and were collected. Azadirachtin (2mg) or [ $^3$ H]-dihydroazadirachtin (1.0 $\mu$ Ci) was added to five separate glass vessels (15ml capacity). Equal volumes (5ml) of the bulk *n*-octanol and distilled water were then added to each vessel. These were sealed using a glass stopper and attached to a rotary shaker. Phases were mixed at 22°C  $\pm$  2°C in the dark for 8 hours. After mixing, the *n*-octanol and distilled water phases were separated by centrifuge (1000rpm for 1 minute) and collected. Azadirachtin within the water phase (5ml) was partitioned into dichloromethane (1ml) using the method described in section 4.4.19. The dichloromethane collections were evaluated for azadirachtin content using the vanillin assay described in Chapter 2. A direct quantification of azadirachtin content within *n*-octanol was not possible. The quantity of azadirachtin within this phase was estimated by comparing the amount of material within the water phase to that originally

introduced. [ $^3\text{H}$ ]-dihydroazadirachtin was analysed within aliquots (50 $\mu\text{l}$ ) of the *n*-octanol and distilled water phases by liquid scintillation counting (see section 4.4.22.). The ratio of azadirachtin or [ $^3\text{H}$ ]-dihydroazadirachtin between phases was logged to give the partition coefficient  $\log P_{\text{ow}}$ .

#### 4.4.6. Determination of compost organic matter and organic carbon content

Four silica basins were placed into a cold muffle furnace at 700°C for 3 hours. After this period they were cooled in a desiccator and weighed. Quantities (approximately 5g) of compost were added to each of the four basins. These were subsequently placed in an oven at 110°C for 12 hours. After this period, basins were removed, cooled in a desiccator and reweighed. The basins containing oven-dried compost were then placed in a furnace and ignited at 550°C for 6 hours. Following this period, the basins containing compost ashes were removed, allowed to cool in a desiccator and reweighed. The weight lost on ignition represented the organic matter content. Organic carbon content was estimated using the Walkley-Black method (Flowers personal communication). This assumes that organic matter contains 58% carbon.

#### 4.4.7. Determination of soil moisture content at a soil water potential of -0.5 bar

The determination of moisture content at a soil water potential of -0.5 bar was carried out using the pressure plate apparatus described by Khan (1987). Soils in replicates of four were placed onto 15 bar ceramic plate extractors (Soil Moisture Equipment Co., Santa Barbara, California), which were flooded with water and allowed to equilibrate for 24 hours. Following this period, excess water was removed and the plates were



placed into a pressure chamber that was adjusted to 0.5 bar using nitrogen gas. Samples were allowed to equilibrate at this pressure for three days, by which time water loss had ceased. The percentage moisture content was determined on an oven-dry basis (110°C).

#### 4.4.8. Adsorption of [ $^3\text{H}$ ]-dihydroazadirachtin to soils and peat

Adsorption studies were conducted using the batch-equilibrium method described by SETAC (1995) guidelines. Soils were sieved to <2mm and air-dried. Peat was used as purchased. [ $^3\text{H}$ ]-dihydroazadirachtin (0.1mCi) was added to five glass vessels (15ml capacity). Distilled water (10ml) and soil or peat (0.5g) was then added to each vessel. Vessels were closed using a glass stopper, attached to a rotary shaker and mixed at  $22^\circ\text{C} \pm 2^\circ\text{C}$  in the dark. After 24 hours, vessels were removed and centrifuged (3000rpm for 3 minutes) at room temperature. The clear supernatant was removed and filtered using a syringe fitted with a 0.2 $\mu\text{m}$  syringe filter. Duplicate aliquots (50 $\mu\text{l}$ ) were taken and analysed for [ $^3\text{H}$ ]-dihydroazadirachtin activity using liquid scintillation counting (see section 4.4.22.). Adsorption was estimated by comparing the quantity of [ $^3\text{H}$ ]-dihydroazadirachtin within the water phase to that originally introduced. Distribution coefficients  $K_d$  ( $K_d = (x/m_s)/C_e$ ) were calculated. The  $K_d$  values were corrected for organic carbon content by calculating the  $K_{oc}$  ( $K_{oc} = K_d/f_{oc}$ )

#### 4.4.9. Desorption of [ $^3\text{H}$ ]-dihydroazadirachtin from soils and peat

Desorption studies were conducted using a modification of the batch-equilibrium method described by SETAC (1995) guidelines. Desorption was measured

immediately after adsorption trials. Soils or peat were removed from the glass vessels used during the adsorption trials, and placed separately into syringes fitted with glass microfibre filter papers (Whatman). These were centrifuged (1000rpm for 20 minutes) to remove water, which was then discarded. Dried soils or peat were then placed into clean glass vessels (15ml capacity) containing fresh distilled water (10ml). Vessels were closed using a glass stopper, attached to a rotary shaker and mixed at  $22^{\circ}\text{C} \pm 2^{\circ}\text{C}$  in the dark. After 24 hours, vessels were removed and analysed using the same methods employed during the adsorption study. Desorption was estimated by comparing the quantity of [ $^3\text{H}$ ]-dihydroazadirachtin within the water phase to that originally introduced.

#### 4.4.10. Breakdown of azadirachtin within soil

Freshly collected soils were sieved to 4mm and adjusted to -0.5 bar moisture content. Weights (50g) of these soils were spread out on aluminium foil. Azadirachtin (25mg) dissolved in acetone (2ml) was distributed over the soil surface and allowed to evaporate. Soils were readjusted to -0.5 bar moisture content and placed into separate, 500ml screw-capped jars to form a 1cm soil layer. After the jars were closed, the soils were thoroughly mixed by rolling for 10 minutes. Incubation jars were stored in the dark at  $22^{\circ}\text{C} \pm 0.01^{\circ}\text{C}$ . The soils were aerated daily and maintained at -0.5 bar moisture content. Samples were taken at 0 days and on a subsequent daily basis for 9 days. At sampling points, soil (5g) was removed. Azadirachtin and breakdown products were extracted into methanol using the method described in section 4.4.20. Methanol collections were evaporated and the dried extracts subsequently taken up in a fresh volume of methanol (2ml). Aliquots (100 $\mu\text{l}$ ) were analysed by HPLC (see

section 4.4.23.) and breakdown quantified by peak area (valley to valley) comparisons. Half-life was calculated from plots of natural log (percentage of material remaining) against time. The main breakdown product was isolated by preparative HPLC (see section 4.4.24.).

#### 4.4.11. Plant growth conditions

Nasturtium seeds were individually sown into pots (6.5cm x 6.5cm x 7cm) containing compost. Plants were grown in a controlled temperature room at  $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , 65% relative humidity and under a photoperiod of 16 hours/day. All trials were conducted under these conditions unless stated. Plants were watered every second day with the following amounts of tap water: Prior to germination, 10ml. Following germination, days 0-10 = 20ml; days 11-20 = 35ml; days 21-30 = 50ml; days 31-40 = 70ml; days 41-60 = 90ml. All trials used this watering regime unless stated.

#### 4.4.12. Root uptake and translocation of [ $^3\text{H}$ ]-dihydroazadirachtin from granules applied to compost

Two application methods were used to study the uptake and translocation of [ $^3\text{H}$ ]-dihydroazadirachtin from sodium alginate granules applied to compost. Nasturtium plants growing in separate pots (6.5cm x 6.5cm x 7cm) that contained compost, were treated with granules (6g). Granules were either sprinkled on to the surface or alternatively mixed homogeneously throughout the compost. Each trial was conducted using eight plants. At 14 days and 28 days after granule application, four plants were cut into sections for extraction. The stem (including petioles) was cut at the point



where it exited the soil and all leaves were removed. Leaves were detached in pairs according to age and these pairs were extracted together (see section 4.4.20). Water was extracted from compost and subsequently filtered using a syringe fitted with a 0.2µm syringe filter. Aliquots (1ml) of plant tissue extracts and compost water were analysed by liquid scintillation counting (see section 4.4.22.). The percentage of water within plant tissues and compost was determined on an oven-dry basis (110°C).

#### 4.4.13. Collection of [<sup>3</sup>H]-dihydroazadirachtin from xylem vessels

Four nasturtium plants (14 days old), growing in compost within separate pots, were de-topped (stems cut 2 cm above the soil surface) under distilled water using a scalpel. After the cut stumps were rinsed with distilled water, an inverted Pasteur pipette tip was placed tightly onto each stump. Distilled water (25ml) containing [<sup>3</sup>H]-dihydroazadirachtin (0.5mCi) was then applied as a drench to the compost within each pot. Exudate (approximately 200µl) accumulating in the tip of the pipette was subsequently collected over a 24-hour period. Duplicate aliquots (100µl) of the exudate were taken and analysed for [<sup>3</sup>H]-dihydroazadirachtin activity by liquid scintillation counting (see section 4.4.22.). A reference application was achieved by removing [<sup>3</sup>H]-dihydroazadirachtin from the above solution.

#### 4.4.14. Collection of [<sup>3</sup>H]-dihydroazadirachtin from phloem vessels

Four nasturtium plants (14 days old) were loaded with [<sup>3</sup>H]-dihydroazadirachtin using the method described in section 4.4.18. After loading, the five oldest leaves per plant were cut under water at the base of their petioles and rinsed. Each leaf was incubated

in a microcentrifuge tube containing either 1.5ml EGTA (1mM) or 1.5ml CaCl<sub>2</sub> (80mM) for 48 hours under continuous light. After this period, the five collection solutions of each replicate were pooled and filtered using a syringe fitted with a 0.2µm syringe filter. Duplicate aliquots (1ml) were taken and analysed for [<sup>3</sup>H]-dihydroazadirachtin activity by liquid scintillation counting (see section 4.4.22.). The presence of [<sup>3</sup>H]-dihydroazadirachtin within phloem vessels was assessed by comparing quantities exuded into each collection solution.

#### 4.4.15. Distribution of [<sup>3</sup>H]-dihydroazadirachtin within the leaf lamina

Nasturtium plants (14 days old) were loaded with [<sup>3</sup>H]-dihydroazadirachtin using the method described in section 4.4.18. After loading, leaves were detached and the abaxial surface exposed to film for 48 hours (see section 4.4.21.).

#### 4.4.16. Metabolism of [<sup>3</sup>H]-dihydroazadirachtin within plants

Seven nasturtium plants (14 days old) were loaded with [<sup>3</sup>H]-dihydroazadirachtin using the method described in section 4.4.18. After loading, plant roots were rinsed under tap water. Plants were then transplanted into peat and immediately watered. Following this initial watering, plants were watered using the regime described in section 4.4.11. At 0 days after loading, and on a subsequent weekly basis for 6 weeks, a single plant was removed and all leaves detached. [<sup>3</sup>H]-dihydroazadirachtin and breakdown products were extracted from leaf material using the method described in section 4.4.20. Duplicate aliquots of the extract were spotted onto TLC plates and

analysed for breakdown (see section 4.4.26.). Half-life was calculated from a plot of natural log (percentage of material remaining) against time.

#### 4.4.17. Specific activity of [ $^3\text{H}$ ]-dihydroazadirachtin

Four nasturtium plants (14 days old) were removed from peat and the roots washed under tap water. The roots of each plant were then submerged into separate solutions of distilled water (10ml) containing azadirachtin and [ $^3\text{H}$ ]-dihydroazadirachtin. Plants were maintained in these conditions for 24 hours under continuous light. After this period, the leaves were detached. Azadirachtin and [ $^3\text{H}$ ]-dihydroazadirachtin were extracted from leaf material using the conditions described in section 4.4.20. Azadirachtin within the loading solution and leaf extracts was quantified by HPLC (see section 4.4.23.). [ $^3\text{H}$ ]-dihydroazadirachtin was quantified by liquid scintillation counting (see section 2.2.22). Specific activity was determined by comparing the ratio of azadirachtin: [ $^3\text{H}$ ]-dihydroazadirachtin within the loading solution to that within the leaf extract.

#### 4.4.18. Loading of Nasturtium plants with [ $^3\text{H}$ ]-dihydroazadirachtin

Nasturtium plants were removed from peat and the roots washed under tap water. Plants were loaded with [ $^3\text{H}$ ]-dihydroazadirachtin by submerging the roots within distilled water (30ml) containing [ $^3\text{H}$ ]-dihydroazadirachtin (0.5mCi) and Tween 20 (0.02% v/v). Plants were maintained in these conditions for 24 hours under continuous light.



#### 4.4.19. Extraction of azadirachtin and [ $^3\text{H}$ ]-dihydroazadirachtin from water

Azadirachtin or [ $^3\text{H}$ ]-dihydroazadirachtin within an aqueous aliquot was partitioned into dichloromethane by vortex mixing for 20 seconds. The resulting mixture was centrifuged (3000rpm for 1 minute) into distinct phases. The hypophase was collected by pipette and the limonoids remaining within the aqueous hyperphase were partitioned into fresh dichloromethane a further three times using this method. The hypophase collections of a replicate were pooled and filtered using a syringe fitted with a 0.2 $\mu\text{m}$  syringe filter.

#### 4.4.20. Extraction of [ $^3\text{H}$ ]-dihydroazadirachtin from soil or plant material

Soil was added to methanol (5ml) and [ $^3\text{H}$ ]-dihydroazadirachtin extracted by vortex mixing for 20 seconds. Leaves or stem material was added to methanol (5ml) and homogenised using an Ultra Turrax (6000rpm for 30 seconds). The resulting extract was spun by centrifuge (3000rpm for 3 minutes) to separate particulate material from the methanol. This methanol phase was collected. The extraction procedure was repeated on the partially extracted material a further 5 times. The extracts of a replicate were pooled and filtered using a syringe fitted with a 0.2 $\mu\text{m}$  syringe filter.

#### 4.4.21. Autoradiography

Autoradiography was conducted by exposure to film (Kodak, MXB film) for an appropriate period at -80°C. Exposure was carried out within cassettes equipped with

intensifier screens. [<sup>3</sup>H]-dihydroazadirachtin was visualised upon developing the film (Kodax, x-omat, 2000 Processor).

#### 4.4.22. Scintillation counting

Liquid scintillation counting was conducted using a Beckman LS 6500 multi-purpose scintillation counter that was programmed to adjust for colour quenching and natural sample luminescence. Disintegrations per minute were recorded over a count time of 3 minutes. Appropriate sample aliquots were added to scintillation fluid (5ml, Ecoscint-A, National Diagnostics) and manually shaken to mix.

#### 4.4.23. Analytical high performance liquid chromatography using the Beckman HPLC system

Analytical reversed-phase HPLC was carried out with a Beckman HPLC system. Samples were injected onto a C18 column (Varian, 4.6 x 250mm, particle size: 5µm) using a Beckman (210A) injector fitted with a 200µl injection loop. Materials eluting from the column were detected using a Beckman (166) programmable detector, set at 217nm that was equipped with a Beckman (406) analogue interface module. Detected peaks were estimated valley to valley and retention times were recorded using a NEC PC-8300 module linked to a Servogor chart recorder. The solvent system consisted of acetonitrile/water in the ratio of 40:60 run isocratically at a flow rate of 1.0ml/min for 30 minutes. There was an average pressure of 207 bar, which was controlled through a Beckman (110B) solvent delivery module. Materials eluting from the column were identified by retention time and comparison with known standards.

#### 4.4.24. Preparative high performance liquid chromatography

Azadirachtin breakdown products were isolated using a modification of the chromatographic conditions described in section 4.4.23. The solvent system was modified and consisted of acetonitrile/water in the ratio of 45:55. Materials eluting from the column were identified and isolated by retention time.

#### 4.4.25. Thin layer chromatography (system 1)

Normal-phase TLC was performed on 5 x 10cm silica gel plates (Alugram, 0.20mm, aluminium backed). Aliquots were spotted onto plates, which were partially pre-run using methanol. Plates were subsequently double developed using a methanol: ethyl acetate (5:95) solvent system for a distance of 7cm.

#### 4.4.26. Thin layer chromatography (system 2)

Normal-phase TLC was performed as described in section 4.4.25. Following development, plates were cut into 15, 5mm sections, and each section analysed by liquid scintillation counting (see section 4.4.22.). Disintegrations per minute within each section were corrected for background activity. Sample breakdown was quantified by comparing disintegrations per minute within each section.



## **4.5. Results**

### **4.5.1. Solubility of azadirachtin and [<sup>3</sup>H]-dihydroazadirachtin**

Azadirachtin had a water solubility that varied between 1.2869 g/l to 1.2941 g/l over the 8-hour study period (figure 4.5.2.). The mean value of 1.2905 g/l was calculated and this represented the water solubility of the limonoid at room temperature.

Azadirachtin and [<sup>3</sup>H]-dihydroazadirachtin partitioned favourably into the lipophilic phase during mixing studies with distilled water and *n*-octanol (figures 4.5.3.A. and 4.5.3.B.). The ratio of this partition was similar for azadirachtin (1:7) and [<sup>3</sup>H]-dihydroazadirachtin (1:7.8). The corresponding *n*-octanol/water partition coefficients, as quantified through the log P<sub>ow</sub>, were consequently also similar. Azadirachtin had a log P<sub>ow</sub> value of 0.85 and [<sup>3</sup>H]-dihydroazadirachtin had a log P<sub>ow</sub> of 0.89.

The specific activity study (figure 4.5.3.C.) revealed that azadirachtin and [<sup>3</sup>H]-dihydroazadirachtin were taken-up by roots and transported to the leaves at almost exactly the same rate. The ratio of azadirachtin: [<sup>3</sup>H]-dihydroazadirachtin remained constant between the root loading solution (1.04μCi/mmol) and the methanol leaf extract (1.08μCi/mmol). This represented a difference of only 4%.

Figure 4.5.1. **Soil characteristics.** (A) Classification of soils used in trials. Data supplied by Hugh Flowers (University of Glasgow). (B) Organic matter, organic carbon and pH (water) of peat. Quantities of compost, in replicates of four, were placed into a cold muffle furnace and ignited at 550°C for 6 hours. The weight lost on ignition represented the organic matter content. Organic carbon content was estimated using the Walkley-Black method, which assumes that organic matter contains 58% carbon. (C) Soil moisture content at a soil water potential of -0.5 bar. Soils in replicates of four were placed onto ceramic plate extractors, which were flooded with water and allowed to equilibrate for 24 hours. Following this period, plates were placed into a pressure chamber that was adjusted to 0.5 bar using nitrogen gas. Samples were allowed to equilibrate at this pressure for three days. The percentage moisture content was determined on an oven dry basis at 110°C.

(A)

	Soil			
Texture	Clay	Silty Clay	Sandy Clay Loam	Sandy Loam
Site	Bank farm	Bank farm	Lennoxtown	Barassie
Land use	Arable farming	Arable farming	Garden	Garden
Soil association	N/A	N/A	Darvel	Dreghorn
Soil series	Midelney	Midelney	Darvel	Dreghorn
Sand, medium + coarse (%)	2.3	1.5	33.5	86
Sand, fine (%)	15.5	7.4	20	1.9
Silt (%)	40.3	50.8	22	10.9
Clay (%)	42.3	40.4	24.4	1.2
Organic carbon (%)	5.9	4.4	3.5	3.3
Organic matter (%)	16.2	14.7	9.1	5.6
pH (water)	7.5	7.5	6.8	7

(B)

	Peat (compost)
Organic carbon (%)	54.4
Organic matter (%)	93.6
pH (water)	5.0

(C)

Soil	Moisture content (%) at -0.5 bar
Clay	27.88 (± 0.48)
Silty Clay	23.23 (± 0.15)
Sandy Clay Loam	27.88 (± 0.11)
Sandy Loam	13.22 (± 0.09)
Peat (compost)	65.36 (± 1.36)



Figure 4.5.2. **Azadirachtin water solubility.** (A) Preliminary water solubility study. Azadirachtin was added to increasing amounts of distilled water at room temperature. The volume of water required to achieve complete dissolution of the sample indicated the approximate solubility. (B) Final water solubility study. Azadirachtin (50mg) was added to three separate glass vessels containing distilled water (10ml). Vessels were sealed and mixed at 30°C. After 1, 5 and 7 hours, a single vessel was removed and allowed to equilibrate at room temperature for 1 hour. The absorbance of an aliquot at 217nm was recorded, and azadirachtin's water solubility at each time interval quantified by the equation  $A = \epsilon \times c \times l$ . The precise solubility at room temperature was determined by taking the mean value of the three assessment points.

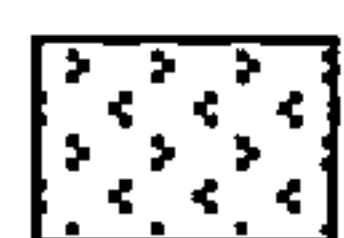
(A)

0.01g azadirachtin soluble in Xml of distilled H <sub>2</sub> O	0.01	0.05	0.1	0.2	1.0	5.0	10
Approx. solubility (g/l)	>1000	1000- 200	200-100	100-50	50-10	50-10	10-1
Complete sample dissolution	No	No	No	No	No	No	Yes

(B)

Time (hours)	2	6	8
Solubility (g/l)	1.2905	1.2941	1.2869
pH of solution	4.98	5.01	4.93

Figure 4.5.3. **Comparison of azadirachtin with [ $^3\text{H}$ ]-dihydroazadirachtin.** (A) Distribution of azadirachtin and [ $^3\text{H}$ ]-dihydroazadirachtin between *n*-octanol and distilled water. Azadirachtin or [ $^3\text{H}$ ]-dihydroazadirachtin, in replicates of five, was mixed between equal volumes of *n*-octanol and distilled water at 22°C for 8 hours. The percentage of azadirachtin or [ $^3\text{H}$ ]-dihydroazadirachtin within each phase was quantified using appropriate methods. Vertical lines represent  $\pm$  S.D. (B) *N*-octanol/water partition coefficient. The ratio of azadirachtin or [ $^3\text{H}$ ]-dihydroazadirachtin between distilled water and *n*-octanol phases was logged to give the partition coefficient  $\log P_{ow}$ . (C) Specific activity of azadirachtin: [ $^3\text{H}$ ]-dihydroazadirachtin. The roots of four nasturtium plants were placed into separate solutions of distilled water containing azadirachtin and [ $^3\text{H}$ ]-dihydroazadirachtin for 24 hours. After this period, leaves were detached and both molecules extracted into methanol. Azadirachtin and [ $^3\text{H}$ ]-dihydroazadirachtin within both the loading solution and leaf extracts were quantified using appropriate methods. Specific activity was determined by comparing the ratio of azadirachtin: [ $^3\text{H}$ ]-dihydroazadirachtin within the loading solution to that within the leaf extract.

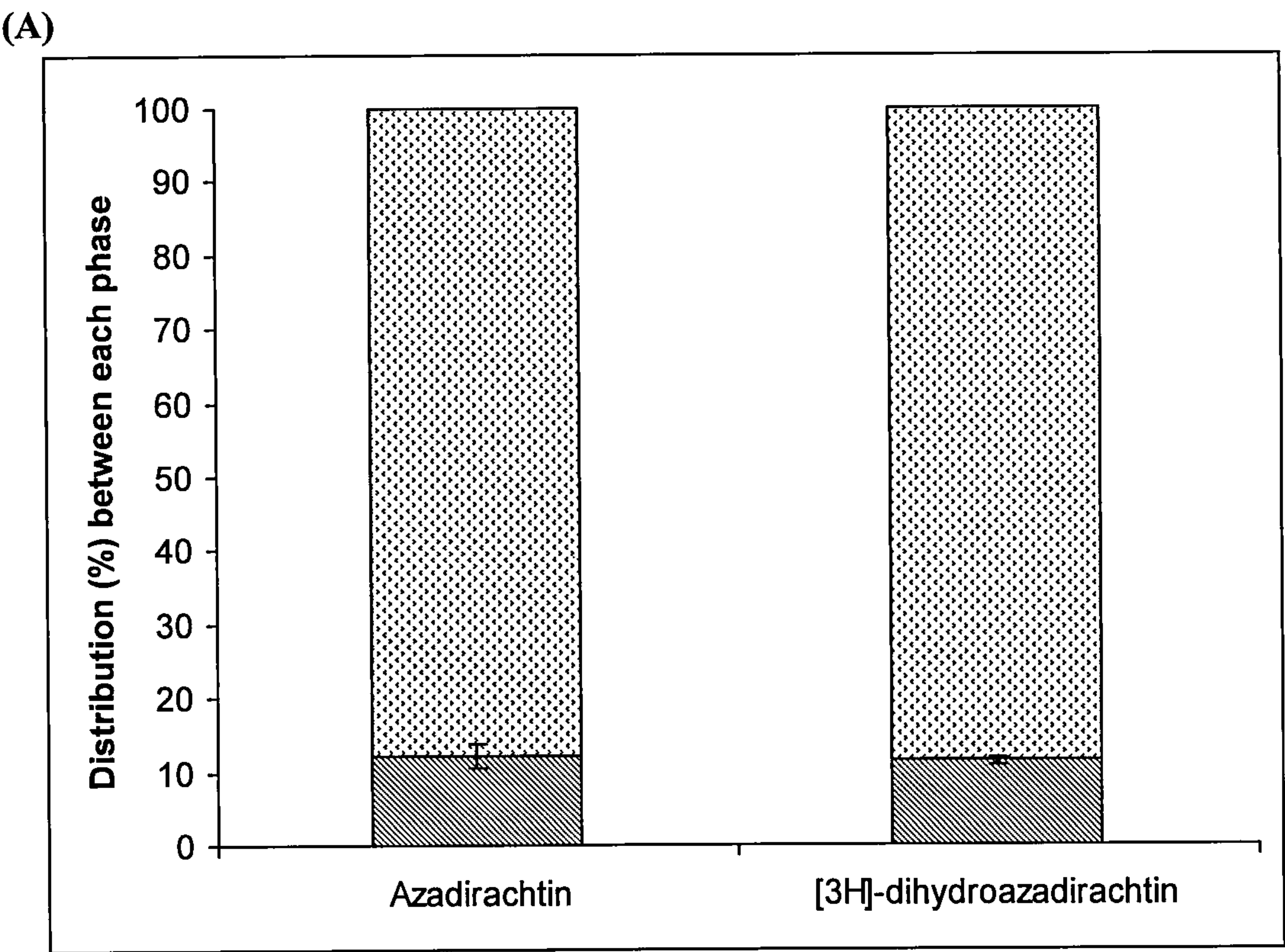


Percentage within *n*-octanol phase



Percentage within water phase





(B)

	Ratio (dH <sub>2</sub> O: Octanol)	Log P <sub>ow</sub>
Azadirachtin	1: 7	0.85
[ <sup>3</sup> H]-dihydroazadirachtin	1: 7.8	0.89

(C)

	Ratio of [ <sup>3</sup> H]-dihydroazadirachtin (μCi): azadirachtin (mmole)
Loading solution	1.04μCi/mmole
Leaf extract solution	1.08μCi/mmole

#### 4.5.2. Aqueous hydrolysis of [ $^3\text{H}$ ]-dihydroazadirachtin

[ $^3\text{H}$ ]-dihydroazadirachtin was hydrolysed at different rates within distilled water buffered at pH 5, pH 7 and pH 9 (figure 4.5.4.). Hydrolysis occurred most rapidly under alkaline conditions (pH 9), where the half-life of [ $^3\text{H}$ ]-dihydroazadirachtin was 7.15 hours. At a neutral pH (pH 7), the half-life was considerably longer at 20.9 days. Finally under acidic conditions, [ $^3\text{H}$ ]-dihydroazadirachtin had the longest half-life of 56.68 days.

#### 4.5.3. Adsorption and desorption of [ $^3\text{H}$ ]-dihydroazadirachtin with soils and peat

[ $^3\text{H}$ ]-dihydroazadirachtin was significantly adsorbed (approximately 50%) only by peat following liquid/solid phase mixing for 24 hours. The four soil types, particularly the sandy loam soil, adsorbed relatively small amounts of limonoid at 10% adsorption or less (figure 4.5.5.A.).

The percentage desorption of [ $^3\text{H}$ ]-dihydroazadirachtin was variable amongst the materials tested. The sandy loam soil was the only material from which complete desorption occurred. The extent of desorption from the other materials ranged from 40% (as with peat) to approximately 90% (as with silty clay). Clay and sandy clay loam all desorbed approximately 50% of the bound [ $^3\text{H}$ ]-dihydroazadirachtin (figure 4.5.5.B.).

The strength of [ $^3\text{H}$ ]-dihydroazadirachtin sorption to soils and peat can be described by the soil sorption coefficient ( $K_d$ ) (figure 4.5.6.). Of the materials tested, the soil

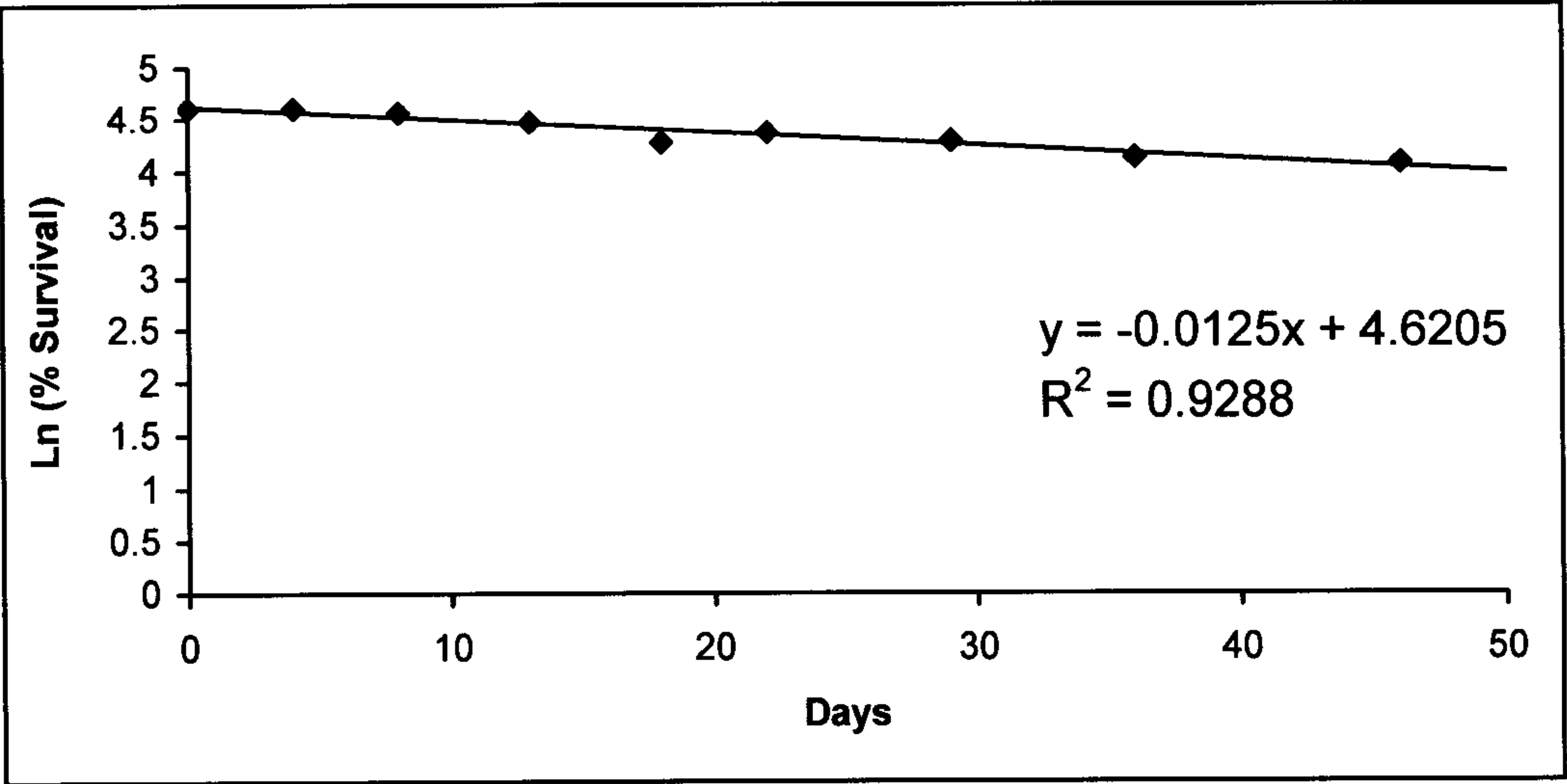
sorption coefficient was highest for peat ( $K_d = 18.23$ ). Consequently, this material bound [ $^3\text{H}$ ]-dihydroazadirachtin the most strongly. In comparison, the strength of sorption of [ $^3\text{H}$ ]-dihydroazadirachtin to soils was considerably less and these materials can be ranked accordingly: sandy loam ( $K_d = 0.6$ ) < sandy clay loam ( $K_d = 1.49$ ) < silty clay ( $K_d = 1.57$ ) < clay ( $K_d = 2.31$ ).

These soil sorption coefficients, corrected for organic carbon content, show that [ $^3\text{H}$ ]-dihydroazadirachtin had a mean  $K_{oc}$  of 33.87 and a mean log  $K_{oc}$  of 1.51.

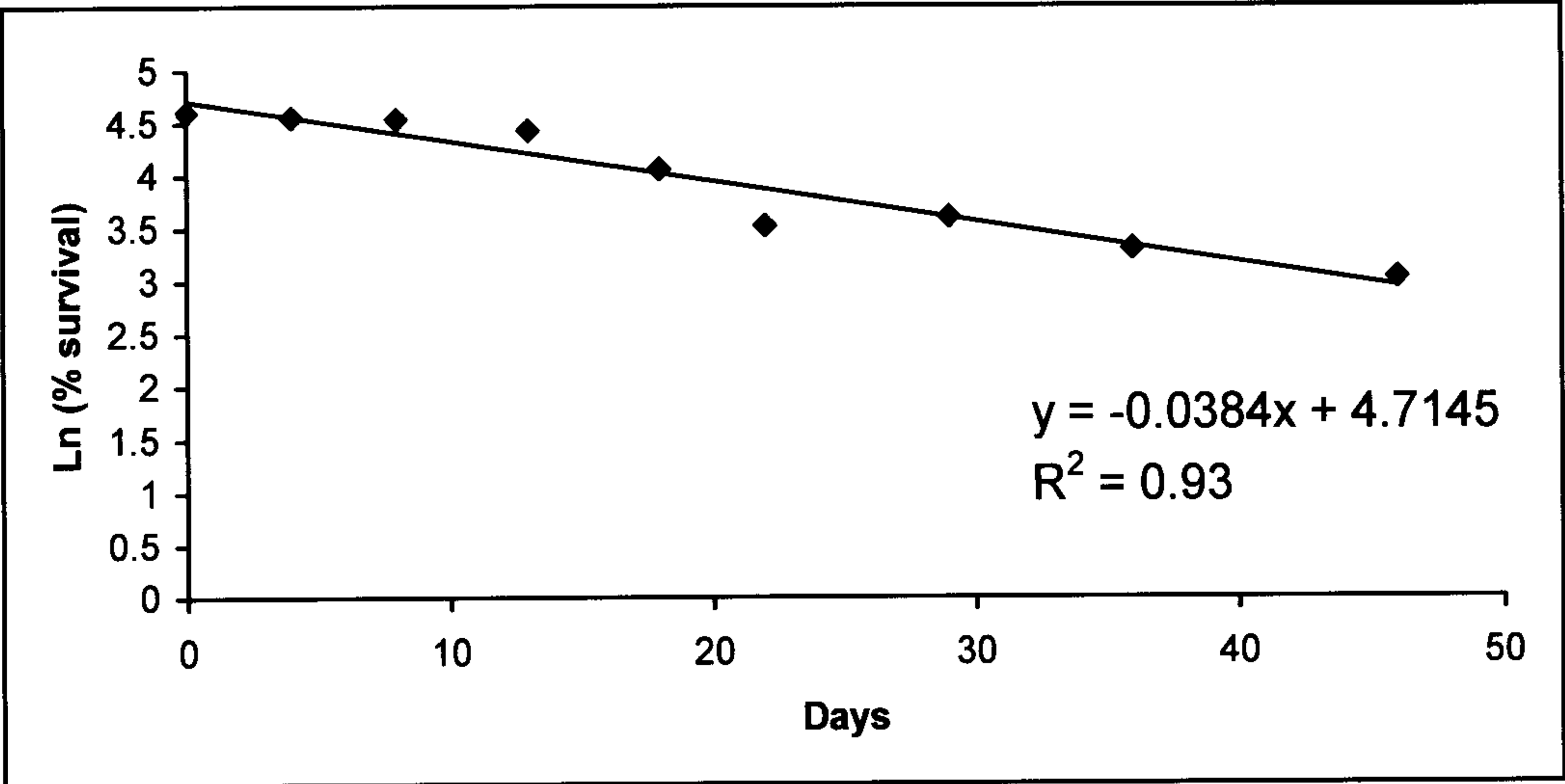


Figure 4.5.4. **Aqueous hydrolysis of [ $^3\text{H}$ ]-dihydroazadirachtin.** Buffer solutions of distilled water at pH 5, pH 7 and pH 9 that contained 0.01% sodium azide were prepared. Volumes were added to separate glass jars that contained [ $^3\text{H}$ ]-dihydroazadirachtin. These jars were sealed and stored in the dark at 22°C. At assessment points, aliquots were removed and [ $^3\text{H}$ ]-dihydroazadirachtin and breakdown products partitioned into dichloromethane. The dichloromethane collections were evaporated and dried extracts were taken up in methanol. Aliquots were analysed by TLC (solvent system = methanol/ethyl acetate at 5:95). Bands were visualised by exposing plates to film for 24 hours. Quantification was achieved using the Image J computer program. Half-life was calculated from plots of natural log (percentage of material remaining) against time. **(A)** Aqueous hydrolysis of [ $^3\text{H}$ ]-dihydroazadirachtin at pH 5 (half-life: 56.68 days). **(B)** Aqueous hydrolysis of [ $^3\text{H}$ ]-dihydroazadirachtin at pH 7 (half-life: 20.90 days). **(C)** Aqueous hydrolysis of [ $^3\text{H}$ ]-dihydroazadirachtin at pH 9 (half-life: 7.15 hours).

(A)



(B)



(C)

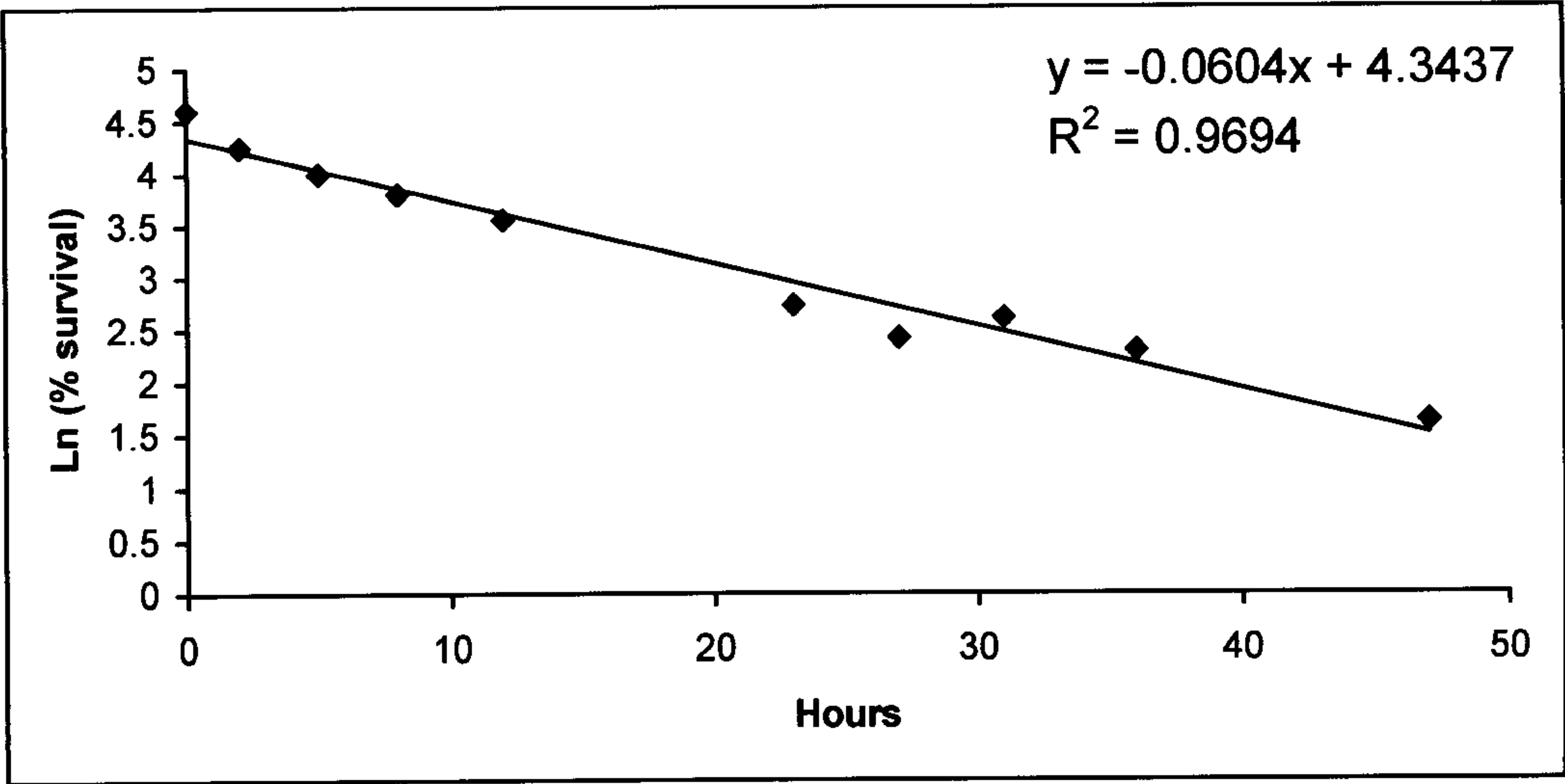
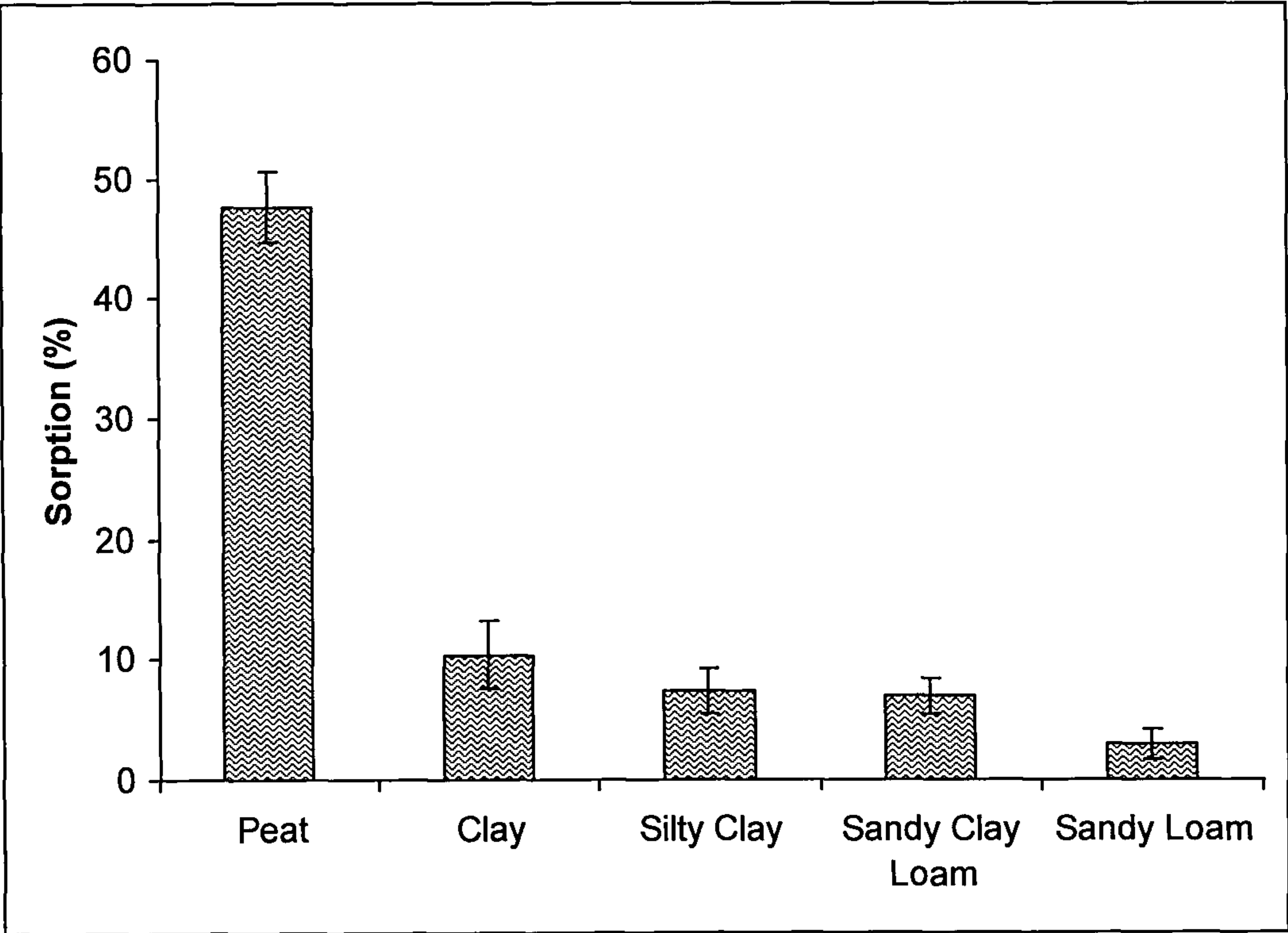


Figure 4.5.5. **Adsorption and desorption of [<sup>3</sup>H]-dihydroazadirachtin with soils and peat.** (A) Soil adsorption. [<sup>3</sup>H]-dihydroazadirachtin, distilled water and soil or peat were mixed together, in replicates of five, at 22°C for 24 hours. After this period, the liquid phase was analysed for [<sup>3</sup>H]-dihydroazadirachtin activity using liquid scintillation counting. Adsorption was estimated by comparing the quantity of [<sup>3</sup>H]-dihydroazadirachtin within the water phase to that originally introduced. (B) Soil desorption. Desorption was measured immediately after adsorption trials. Soils or peat were spun dry by centrifuge and mixed with fresh volumes of distilled water at 22°C for 24 hours. Liquid phases were analysed using the same methods employed during the adsorption study. Desorption was estimated by comparing the quantity of [<sup>3</sup>H]-dihydroazadirachtin within the water phase to that originally introduced. Vertical lines represent  $\pm$  S.D.



(A)



(B)

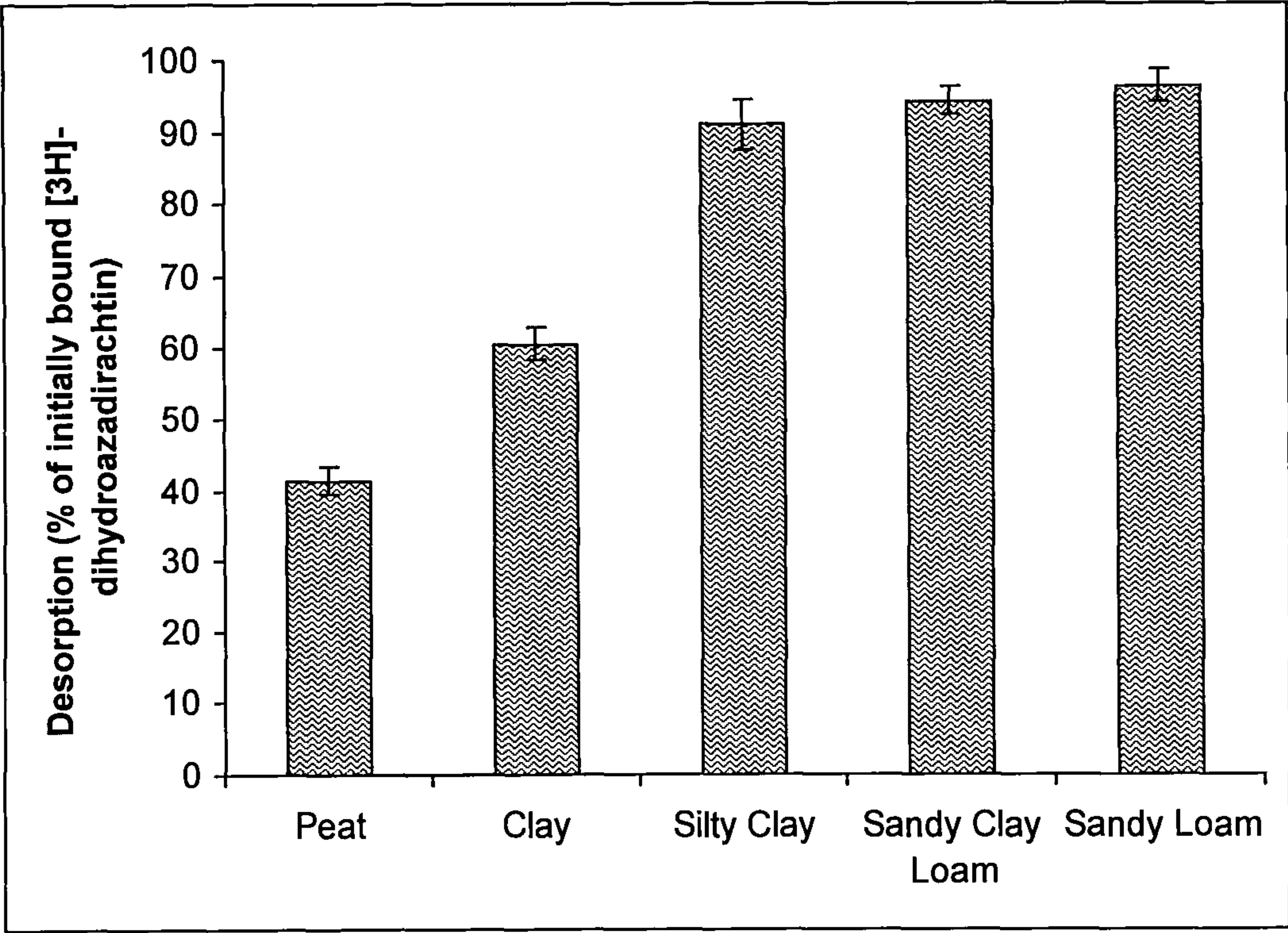


Figure 4.5.6. **Soil sorption and soil organic carbon sorption coefficients for [<sup>3</sup>H]-dihydroazadirachtin.** Adsorption studies were conducted using the batch-equilibrium method described within the text. Adsorption was estimated by comparing the quantity of [<sup>3</sup>H]-dihydroazadirachtin within the water phase to that originally introduced. Distribution coefficients  $K_d$  ( $K_d = (x/m_s)/C_e$ ) were calculated where  $x/m_s$  is the concentration of pesticide in the solid phase and  $C_e$  is the equilibrium pesticide solution concentration. The  $K_d$  values were corrected for organic carbon content by calculating the  $K_{oc}$  ( $K_{oc} = K_d/f_{oc}$ )

Material	Ratio (water: solid phase)	K <sub>d</sub>	Log K <sub>d</sub>	K <sub>oc</sub>	Log K <sub>oc</sub>
Peat	52:48	18.23	1.26	33.52	1.53
Clay	90:10	2.31	0.36	39.18	1.59
Silty Clay	93:7	1.57	0.20	35.73	1.55
Sandy Clay Loam	93:7	1.49	0.17	42.63	1.63
Sandy Loam	97:3	0.60	-0.22	18.30	1.26



#### 4.5.4. Breakdown of azadirachtin within soil

Following addition to soil, azadirachtin was degraded into a single, more polar breakdown product (figure 4.5.7.). This material was isolated by preparative HPLC (figure 4.5.10.) and identified as 3-deacetylazadirachtin by NMR (result not presented).

Azadirachtin within soils was found to degrade rapidly, with half-lives ranging from approximately one to twelve days depending on the soil type (figures 4.5.8. and 4.5.9.). The shortest half-life of azadirachtin was shown to be within in the sandy clay loam soil (half-life = 1.06 days), while in contrast the half-life of the limonoid was longest in the sandy loam soil (half-life = 11.9 days).

The plots of natural log (% survival) against days indicate that the speed of azadirachtin degradation within all soils appeared to increase with time (figures 4.5.8.A., 4.5.8.C., 4.5.9.A. and 4.5.9.C.). A single linear trendline initially applied to each data set were shown to provide poor fits. Therefore, two trendlines were applied to the data to account for biphasic degradation. Figures 4.5.8.B., 4.5.8.D., 4.5.9.B. and 4.5.9.D. show the half-life of azadirachtin within each of the soils following biphasic breakdown. The equation of the trendlines used to calculate these rates and the corresponding  $R^2$  value are also shown on the tables. This data show that a good linearity was obtained in the rate of breakdown, by separating the plots into days 0-6 ( $R^2 = 0.9583$ ) and days 6-9 ( $R^2 = 0.9985$ ) for sandy loam soil, days 0-3 ( $R^2 = 0.9406$ ) and days 3-7 ( $R^2 = 0.9885$ ) for the sandy clay loam soil, days 0-5 ( $R^2 = 0.9708$ ) and days 5-9 ( $R^2 = 0.9586$ ) for the clay soil and finally days 0-5 ( $R^2 = 0.9915$ ) and days

5-9 ( $R^2 = 0.9429$ ) for the silty clay soil. Consequently, based on this interpretation, azadirachtin had a half-life of 11.9 days during the first six days in the sandy loam soil. From day 6 to day 9, the half-life of the limonoid was reduced to 2.70 days. The half-life of azadirachtin within the sandy clay loam soil was 6.17 days and 1.06 days between days 0-3 and days 3-7 respectively. Azadirachtin within the clay soil had a half-life of 3.74 days from days 0-5 and half-life of 1.43 days from days 5-9. Finally, within the silty clay soil, the half-life of azadirachtin was 5.27 days from days 0-5 and 2.06 days during days 5-9.

#### 4.5.5. Root uptake and translocation of [ $^3\text{H}$ ]-dihydroazadirachtin from soil applied granules

Sodium alginate granules applied to compost, as either a surface treatment or homogeneous mix, released [ $^3\text{H}$ ]-dihydroazadirachtin into the compost water. The radiolabelled tracer was subsequently absorbed by roots from solution, and accumulated by aerial regions of the plant (figure 4.5.11.). The concentration found within leaves was initially influenced by the method of granule application. At 14 days after application, the highest concentration of [ $^3\text{H}$ ]-dihydroazadirachtin within leaves was achieved by applying granules as a mix throughout the compost. However, there was no difference between the two application methods, in terms of concentrations accumulated by leaves, after 28 days.

Granule application method did not influence the concentrations of [ $^3\text{H}$ ]-dihydroazadirachtin recorded within compost water at 14-day and 28-day assessments.

Within the plant, a concentration gradient of [ $^3\text{H}$ ]-dihydroazadirachtin existed. The leaves that were lowest on the stem, and consequently the oldest, contained the highest concentrations of the tracer. Leaves at higher stem positions contained decreasing amounts of radioactivity. The concentration of [ $^3\text{H}$ ]-dihydroazadirachtin within the stem water was considerably less than levels found within the leaf material.

When granules were applied as a homogeneous mix, the concentration of [ $^3\text{H}$ ]-dihydroazadirachtin at 14 days and 28 days within the compost water and eldest leaf pair was the same. All younger leaves contained lower levels. However, this was not the case following a surface application of granules. At 14 days after granule application, there was a considerably greater amount of [ $^3\text{H}$ ]-dihydroazadirachtin in the compost water. However, after 28 days, concentrations were approximately equal between the eldest leaf pair and compost water. All younger leaves contained lower levels of [ $^3\text{H}$ ]-dihydroazadirachtin.

Between the 14-day and 28-day assessments, the concentration of [ $^3\text{H}$ ]-dihydroazadirachtin within leaf, stem and compost water did not increase when granules were applied as a homogeneous mix. However, following a surface application of granules, the concentration within the leaves increased during this period, although the levels within the stem and compost water remained relatively unchanged.



Figure 4.5.7. **Breakdown of azadirachtin within soils (1).** Azadirachtin was added to weights of freshly collected soils, which were subsequently stored in the dark at 22°C. Soils were aerated daily and maintained at -0.5 bar moisture content. At sampling points a quantity of soil was removed. Azadirachtin and breakdown products were extracted into methanol. Aliquots were analysed using the Beckman HPLC system as described in section 4.4.23. (column = C18, solvent system = acetonitrile/water at 40:60). **(A)** Typical HPLC chromatogram of azadirachtin after 0 days within the sandy clay loam (Darvel) soil. **(B)** Typical HPLC chromatogram of azadirachtin and main breakdown product after 4 days within the sandy clay loam (Darvel) soil. **(C)** Typical HPLC chromatogram of azadirachtin and main breakdown product after 9 days within the sandy clay loam (Darvel) soil.

Key: I.P. = Injection point

Unknown = Main azadirachtin breakdown product (Retention time: 7.7min)

AZA = Azadirachtin-A (Retention time: 14.5min)

The peaks immediately following the injection point were also present in blank soil extracts.

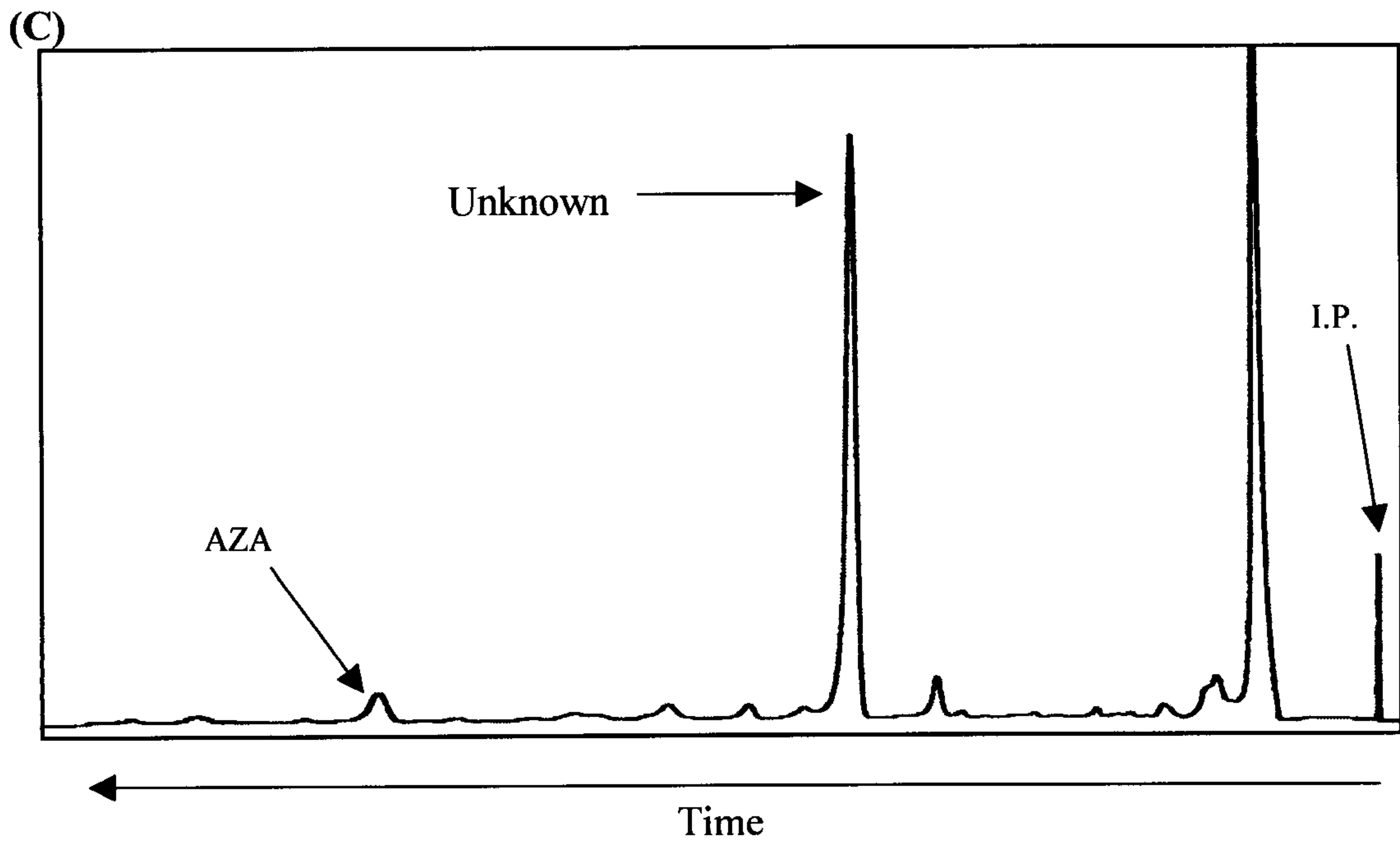
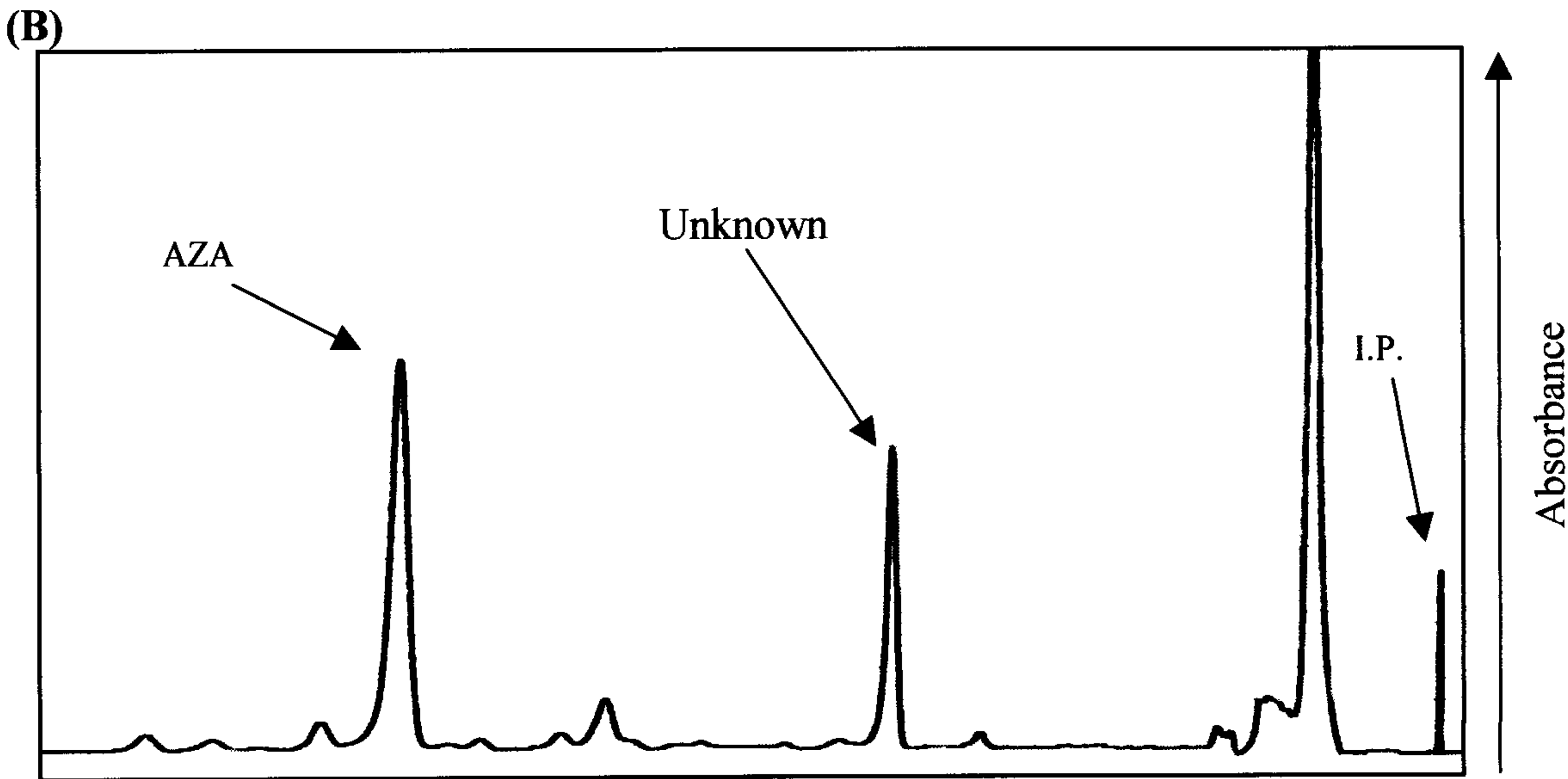
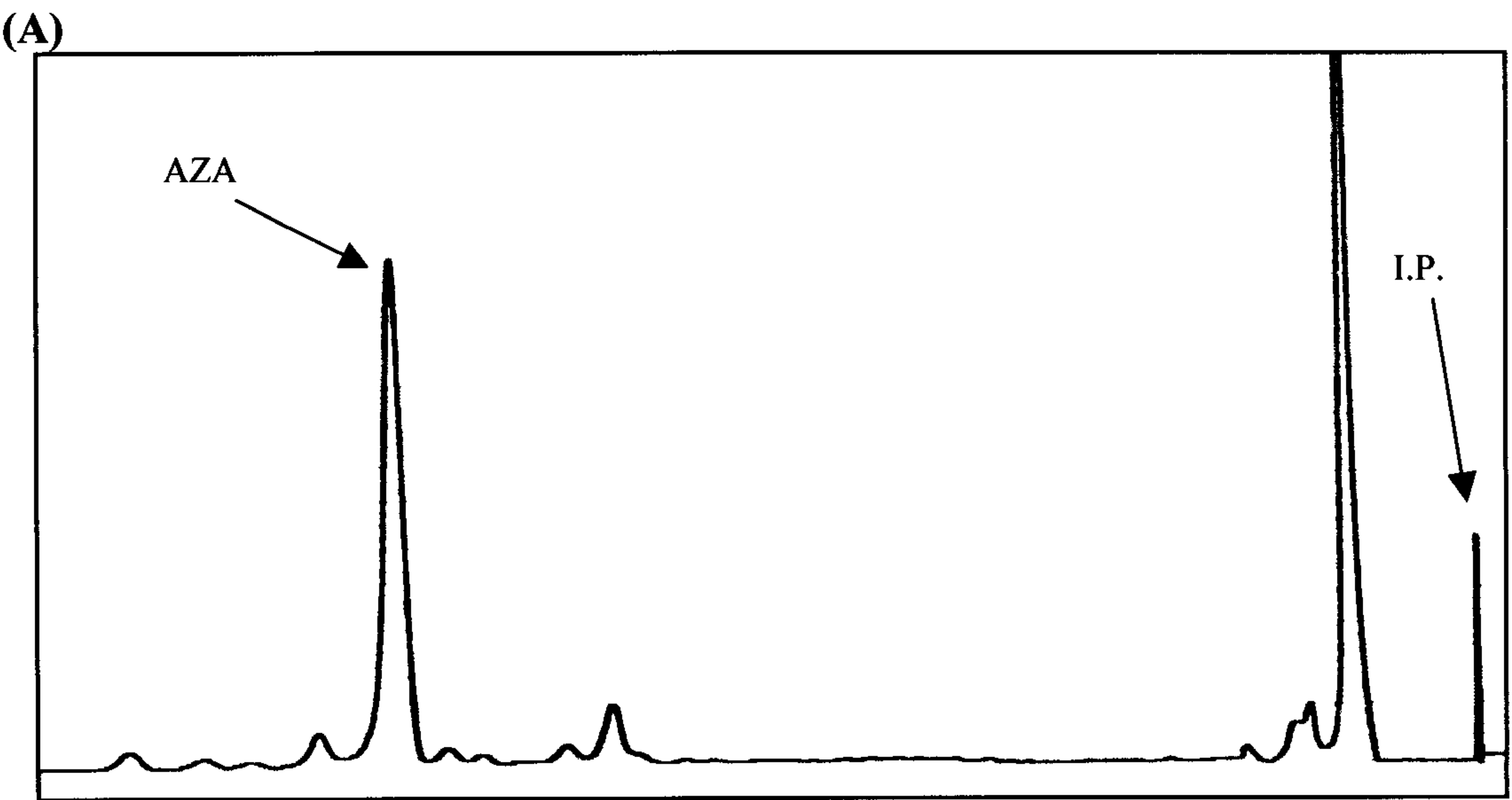
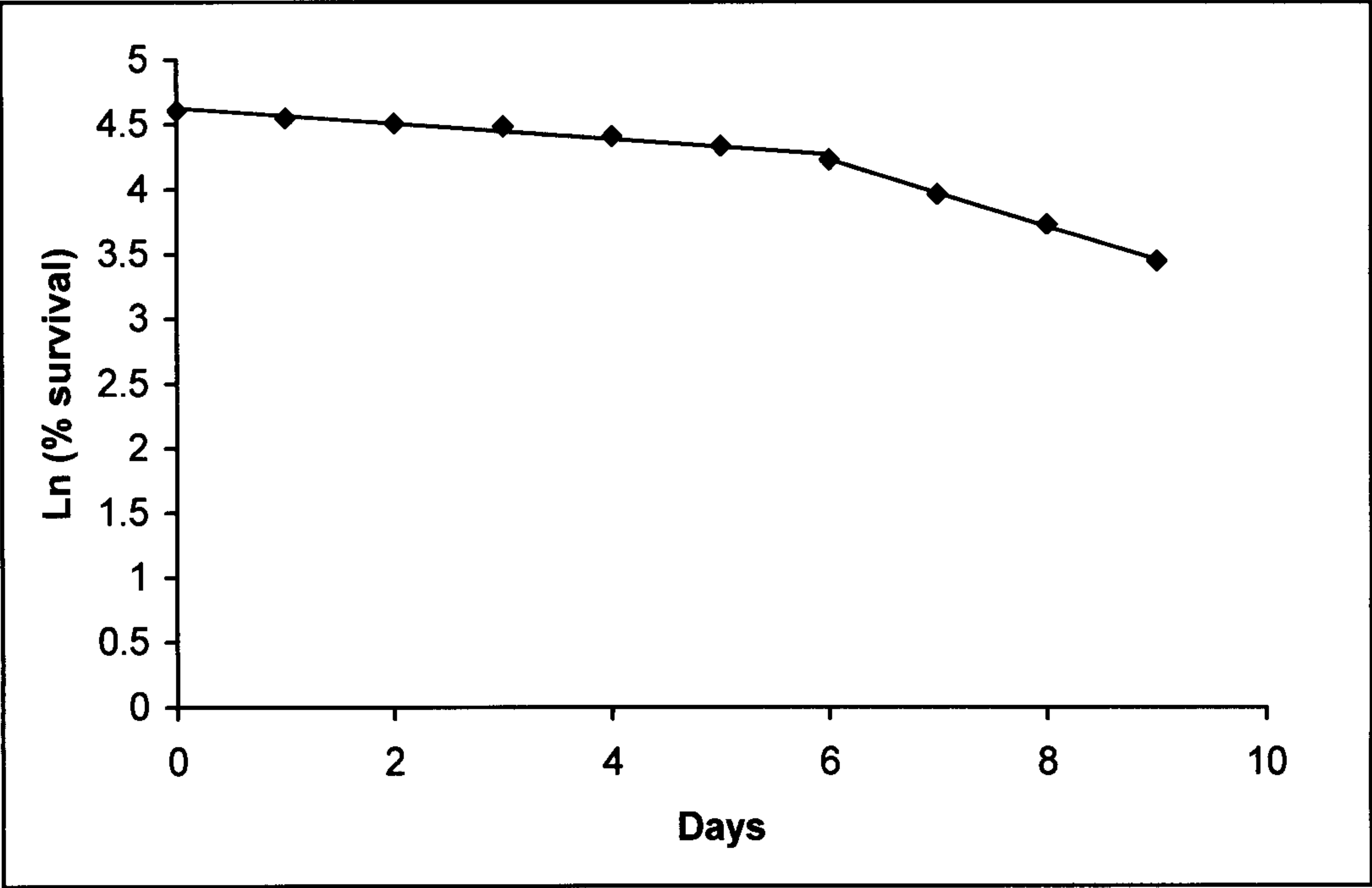


Figure 4.5.8. **Breakdown of azadirachtin within soils (2).** Azadirachtin was added to weights of freshly collected soils, which were subsequently stored in the dark at 22°C. Soils were aerated daily and maintained at -0.5 bar moisture content. At sampling points a quantity of soil was removed. Azadirachtin and breakdown products were extracted into methanol. Aliquots were analysed using the Beckman HPLC system as described in section 4.4.23. (column = C18, solvent system = acetonitrile/water at 40:60). Breakdown was quantified by peak area (valley to valley) comparisons. Half-life was calculated from plots of natural log (percentage of material remaining) against time. **(A)** The degradation of azadirachtin within a sandy loam soil. **(B)** The change in the half-life of azadirachtin within a sandy loam soil between days 0-6 and days 6-9. Biphasic interpretation. **(C)** The degradation of azadirachtin within a sandy clay loam soil. **(D)** The change in the half-life of azadirachtin within sandy clay loam between days 0-3 and days 3-7. Biphasic interpretation.

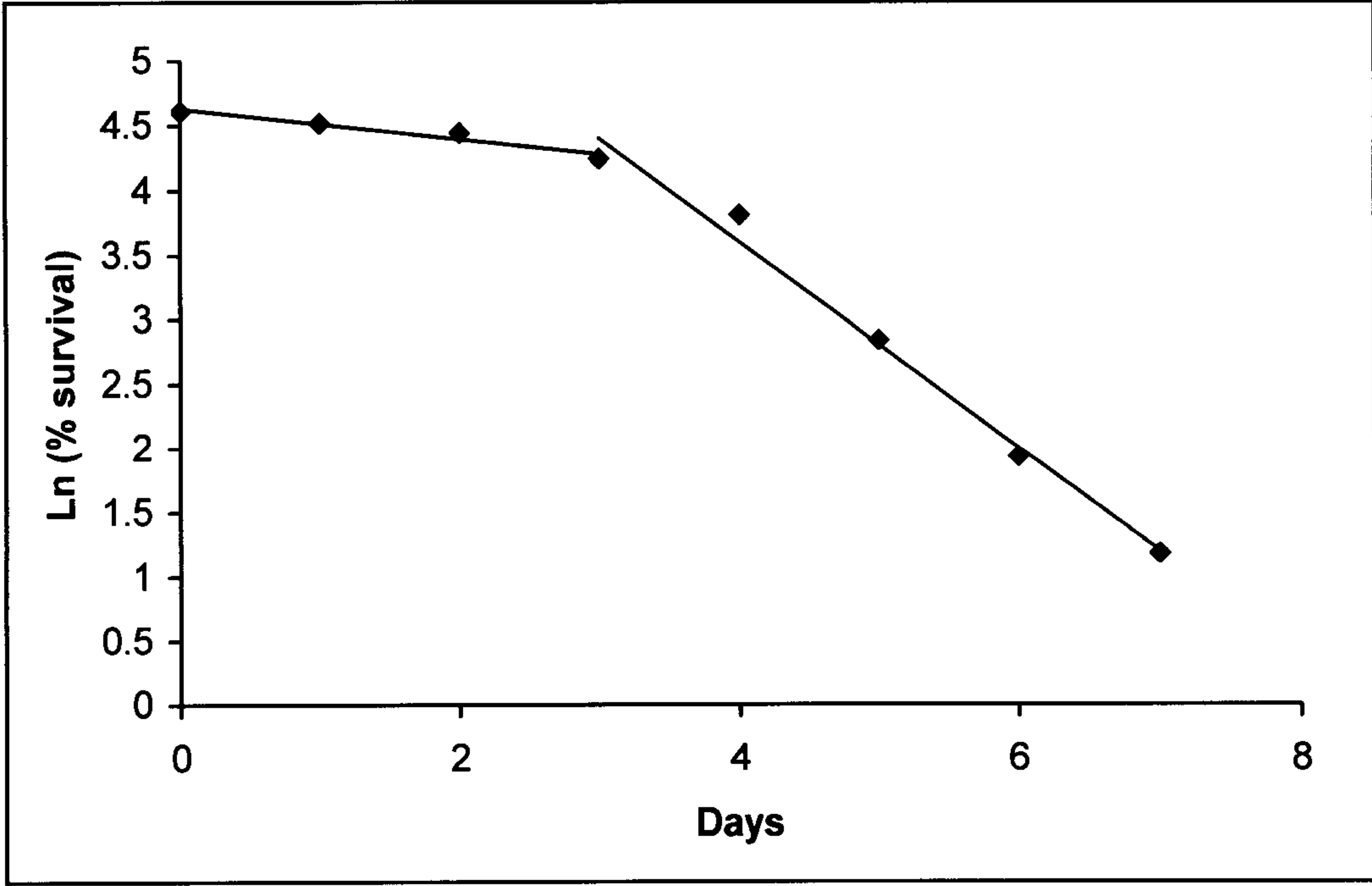
(A)



(B)

Days	Equation	R <sup>2</sup> value	Half-life (days)
0-6	y = -0.0597x + 4.6225	0.9583	11.9
6-9	y = -0.2564x + 4.2248	0.9985	2.70

(C)



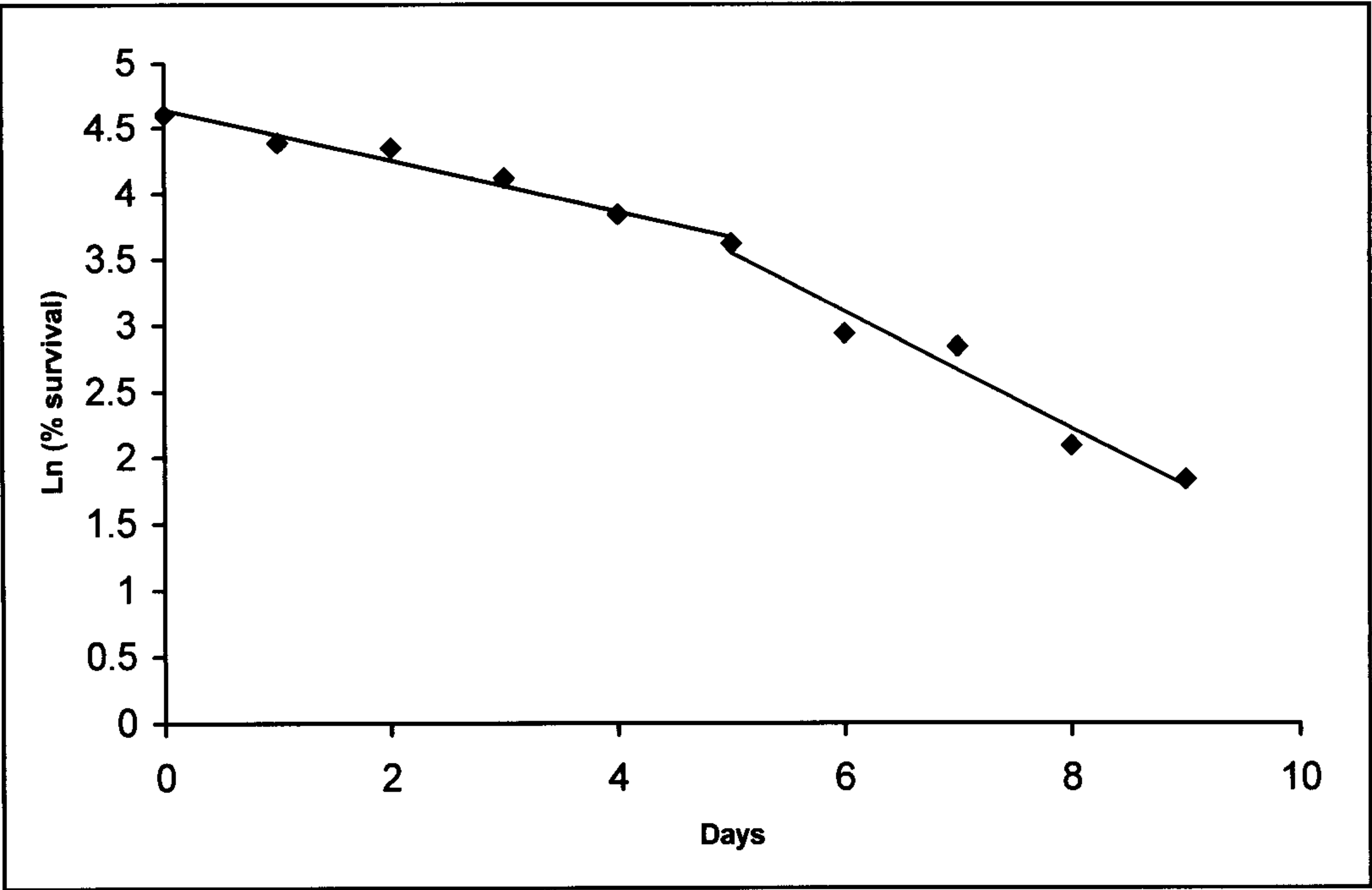
(D)

Days	Equation	R <sup>2</sup> value	Half-life (days)
0-3	y = -0.1157x + 4.6263	0.9406	6.17
3-7	y = -0.8008x + 4.4016	0.9885	1.06



Figure 4.5.9. **Breakdown of azadirachtin within soils (3).** Azadirachtin was added to weights of freshly collected soils, which were subsequently stored in the dark at 22°C. Soils were aerated daily and maintained at -0.5 bar moisture content. At sampling points a quantity of soil was removed. Azadirachtin and breakdown products were extracted into methanol. Aliquots were analysed using the Beckman HPLC system as described in section 4.4.23. (column = C18, solvent system = acetonitrile/water at 40:60). Breakdown was quantified by peak area (valley to valley) comparisons. Half-life was calculated from plots of natural log (percentage of material remaining) against time. **(A)** The degradation of azadirachtin within a clay soil. **(B)** The change in the half-life of azadirachtin within a clay soil between days 0-5 and days 5-9. Biphasic interpretation. **(C)** The degradation of azadirachtin within a silty clay soil. **(D)** The change in the half-life of azadirachtin within a silty clay soil between days 0-5 and days 5-9.

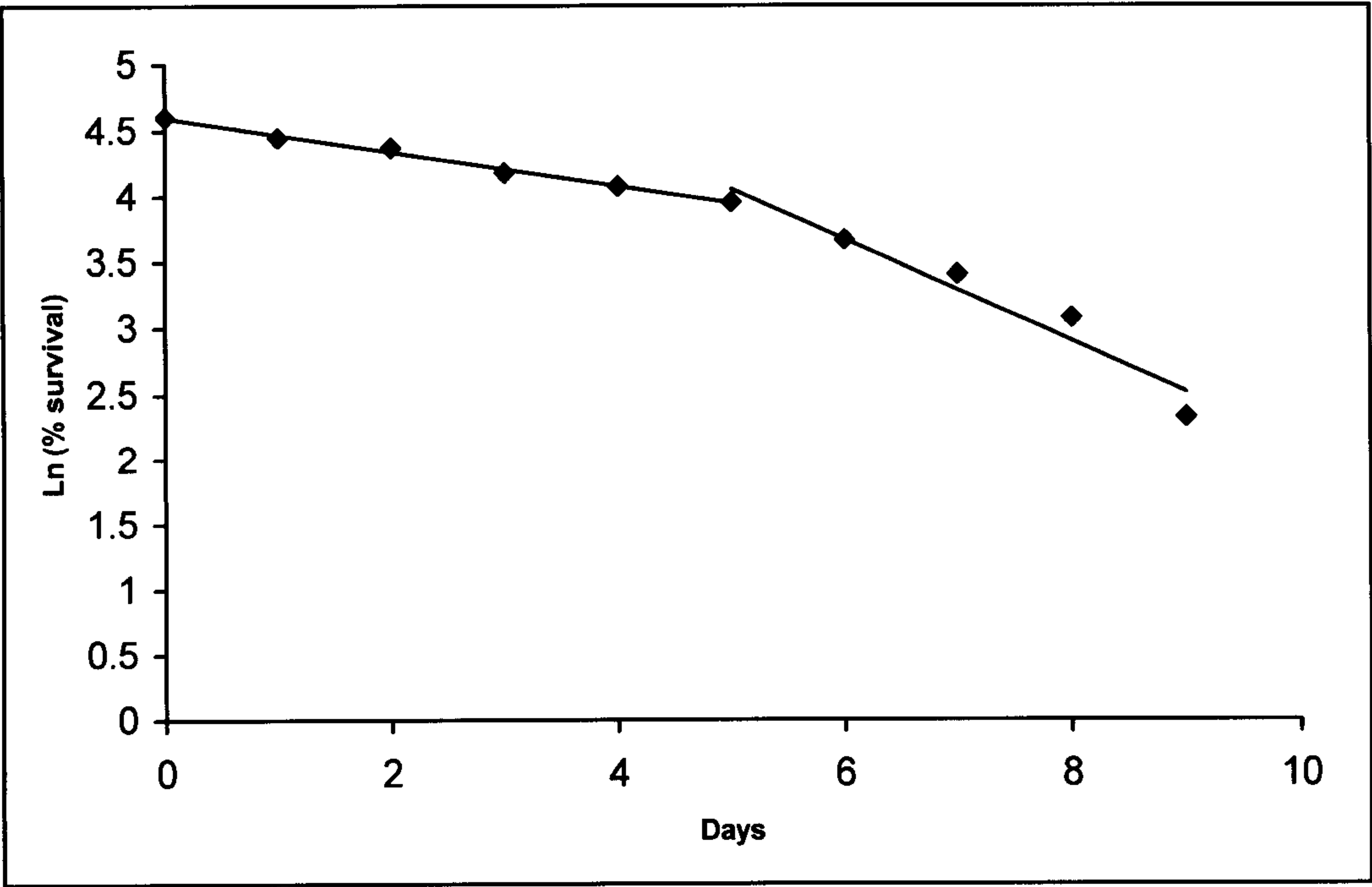
(A)



(B)

Days	Equation	R <sup>2</sup> value	Half-life (days)
0-5	y = -0.1943x + 4.638	0.9708	3.74
5-9	y = -0.4403x + 3.5477	0.9586	1.43

(C)



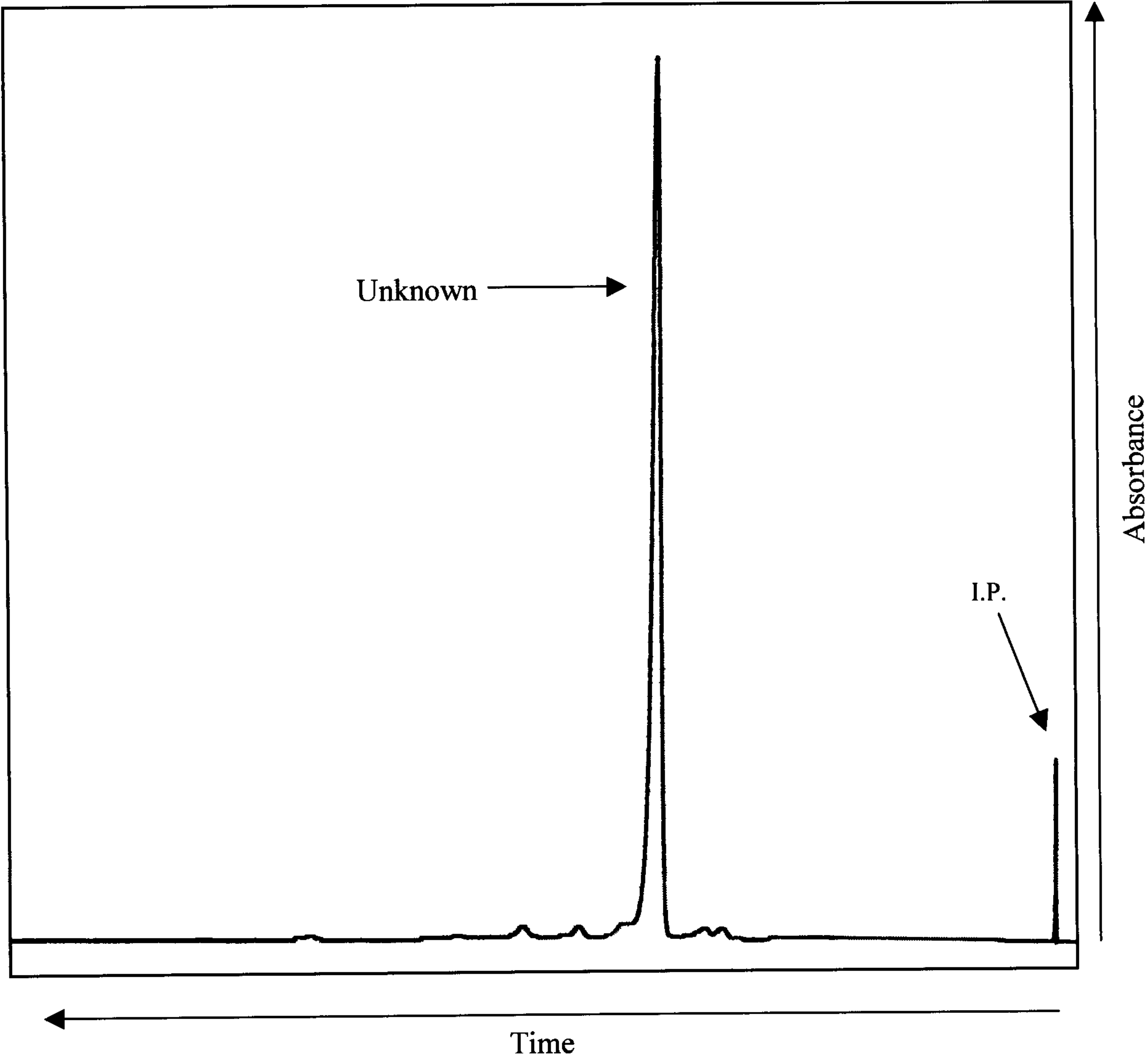
(D)

Days	Equation	R <sup>2</sup> value	Half-life (days)
0-5	y = -0.1301x + 4.5987	0.9915	5.27
5-9	y = -0.3842x + 4.0561	0.9429	2.06

**Figure 4.5.10. Isolation of the main azadirachtin breakdown product.** Breakdown studies were conducted using the methods described in the text. Azadirachtin and breakdown products were extracted from soil into methanol. Methanol extracts were analysed using the Beckman HPLC system as described in section 4.4.23. (column = C18). The main breakdown product was identified and collected according to retention time. During analytical HPLC the solvent system consisted of acetonitrile/water in the ratio of 40:60 run isocratically. During preparative HPLC the solvent system consisted of acetonitrile/water in the ratio of 45:55 run isocratically.

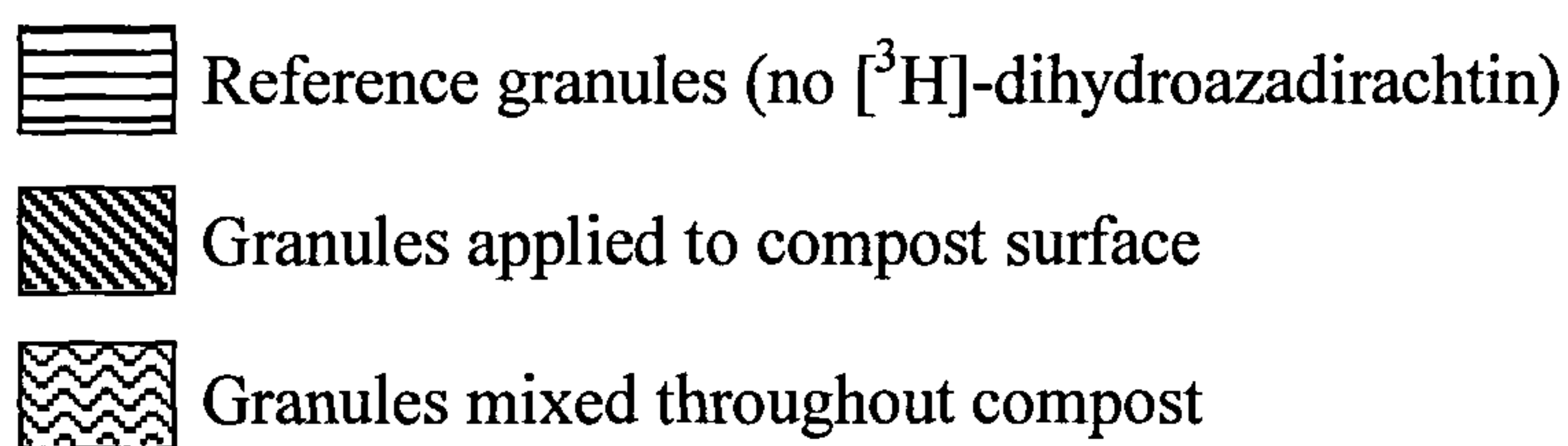
Key: I.P. = Injection point

Unknown = Main azadirachtin breakdown product (Retention time: 7.7min)



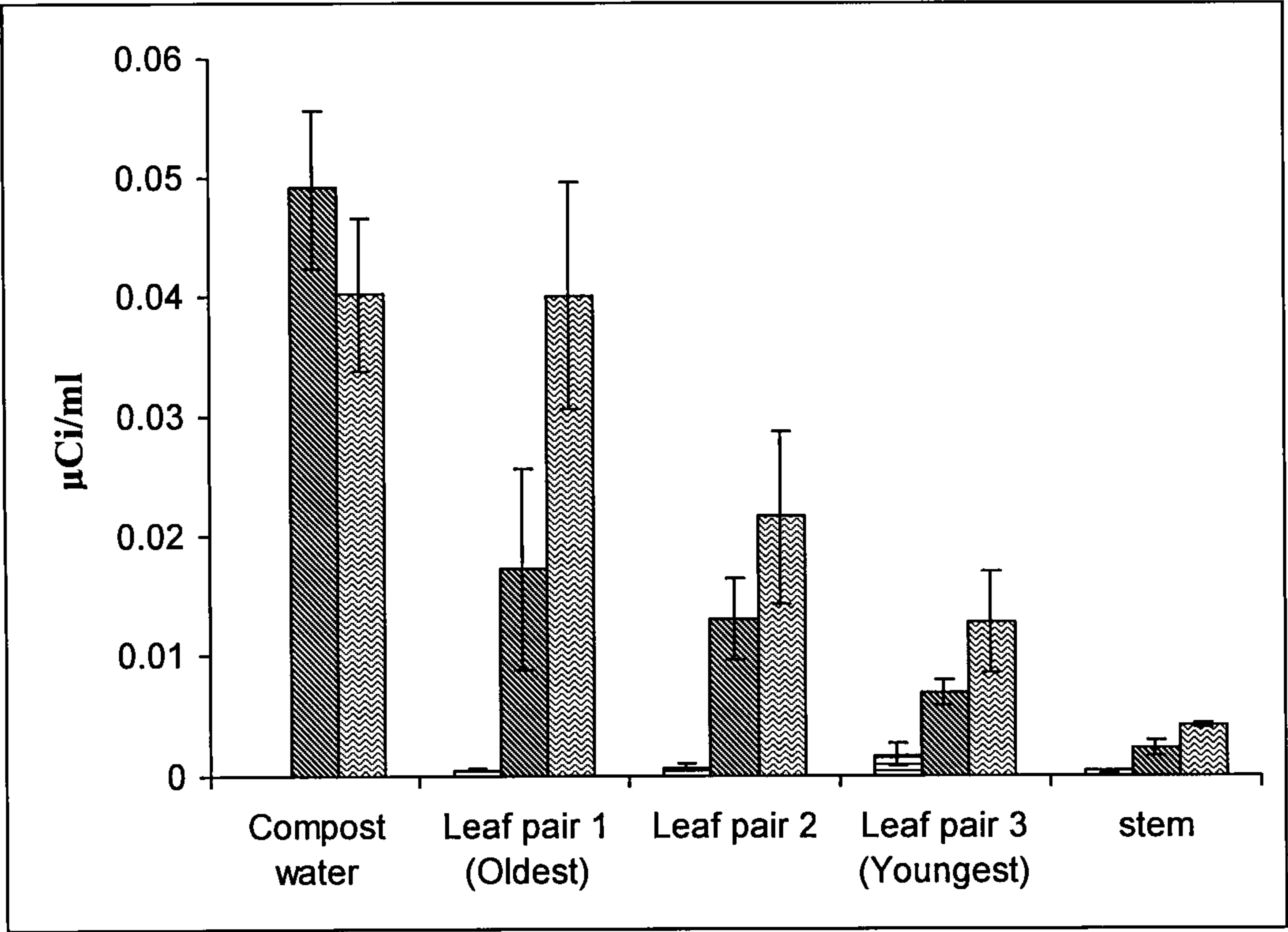


**Figure 4.5.11. Root uptake and translocation of [ $^3\text{H}$ ]-dihydroazadirachtin from soil applied granules.** Nasturtium plants were treated with granules containing [ $^3\text{H}$ ]-dihydroazadirachtin. Granules were either sprinkled on to the surface or alternatively mixed homogeneously throughout the compost. Each trial was conducted using eight plants. At 14 days and 28 days after granule application, four plants were cut into sections. The stem (including petioles) was cut at the point where it exited the soil and all leaves were removed. Leaves were detached in pairs according to age and these pairs were extracted together. Water was extracted from compost. Aliquots of the plant tissue extracts and compost water were analysed by liquid scintillation counting. The percentage of water within plant tissues and compost was determined on an oven dry basis ( $110^\circ\text{C}$ ). **(A)** Concentration of [ $^3\text{H}$ ]-dihydroazadirachtin within soil and plant systems 14 days after granule application. **(B)** Concentration of [ $^3\text{H}$ ]-dihydroazadirachtin within soil and plant systems 28 days after granule application. Vertical lines represent  $\pm$  S.D.

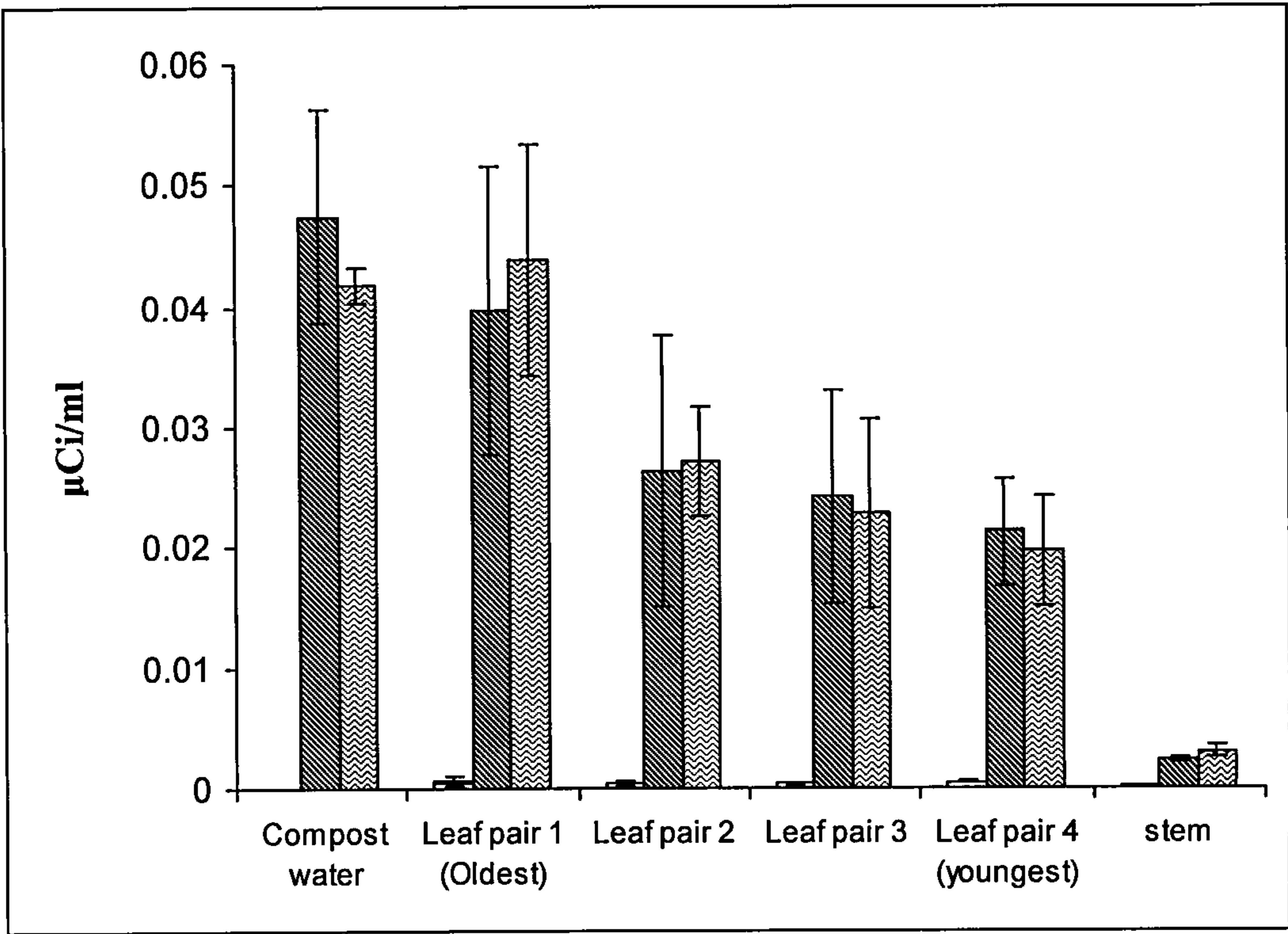


Higher leaf pair numbers represent leaves that are younger and are positioned higher on the plant stem. An extraction of radioactivity from roots and compost solids was not possible.

(A)



(B)



#### 4.5.6. The behaviour of [ $^3\text{H}$ ]-dihydroazadirachtin within plants

Following the treatment of plant roots with solutions containing [ $^3\text{H}$ ]-dihydroazadirachtin, considerable quantities of the tracer were recovered from the xylem exudate of de-topped individuals (figure 4.5.12.A.). Both phloem exudate collection solutions contained significant quantities of [ $^3\text{H}$ ]-dihydroazadirachtin. However, the amounts of tracer exuded were significantly greater ( $p < 0.05$ ) when EGTA instead of  $\text{CaCl}_2$  was used for collection (figure 4.5.12.B.). This comparison suggested that [ $^3\text{H}$ ]-dihydroazadirachtin was present within the phloem.

Autoradiographs (figure 4.5.13.) indicated the distribution of [ $^3\text{H}$ ]-dihydroazadirachtin throughout the leaf lamina, following root uptake from aqueous solutions. Films showed that the tracer was more or less evenly distributed throughout the leaf tissue. However, there appeared to be some increased accumulation around leaf edges, while there was no apparent build up within leaf veins.

Stability studies indicated that [ $^3\text{H}$ ]-dihydroazadirachtin was relatively stable within plant tissues (figure 4.5.14.). Following root up-take from an aqueous loading solution, the tracer had a half-life of 65 days.

Figure 4.5.12. **Transport of [ $^3\text{H}$ ]-dihydroazadirachtin within the vascular systems of plants.** (A) Collection of [ $^3\text{H}$ ]-dihydroazadirachtin from xylem vessels. Four nasturtium plants growing in compost, were de-topped. Distilled water containing [ $^3\text{H}$ ]-dihydroazadirachtin was then applied as a drench to the compost. Exudate from cut stumps was collected over a 24-hour period. Aliquots of the exudate were analysed for [ $^3\text{H}$ ]-dihydroazadirachtin activity by liquid scintillation counting. A reference application was achieved by removing [ $^3\text{H}$ ]-dihydroazadirachtin from the above solution. (B) Collection of [ $^3\text{H}$ ]-dihydroazadirachtin from phloem vessels. Four nasturtium plants were loaded with [ $^3\text{H}$ ]-dihydroazadirachtin. After loading, leaves were cut at the base of their petioles. Each leaf was incubated in a microcentrifuge tube containing either EGTA or  $\text{CaCl}_2$  for 48 hours. After this period, the collection solutions of each replicate were pooled and filtered. Aliquots were analysed for [ $^3\text{H}$ ]-dihydroazadirachtin activity by liquid scintillation counting. The presence of [ $^3\text{H}$ ]-dihydroazadirachtin within phloem vessels was assessed by comparing quantities exuded into each collection solution. Vertical lines represent  $\pm$  S.D.



(A)

	$\mu\text{Ci}/50\mu\text{l}$ xylem exudate
Reference	$1.33 \times 10^{-6} (\pm 2.75 \times 10^{-7})$
Treatment	$5.77 \times 10^{-3} (\pm 1.56 \times 10^{-3})$

(B)

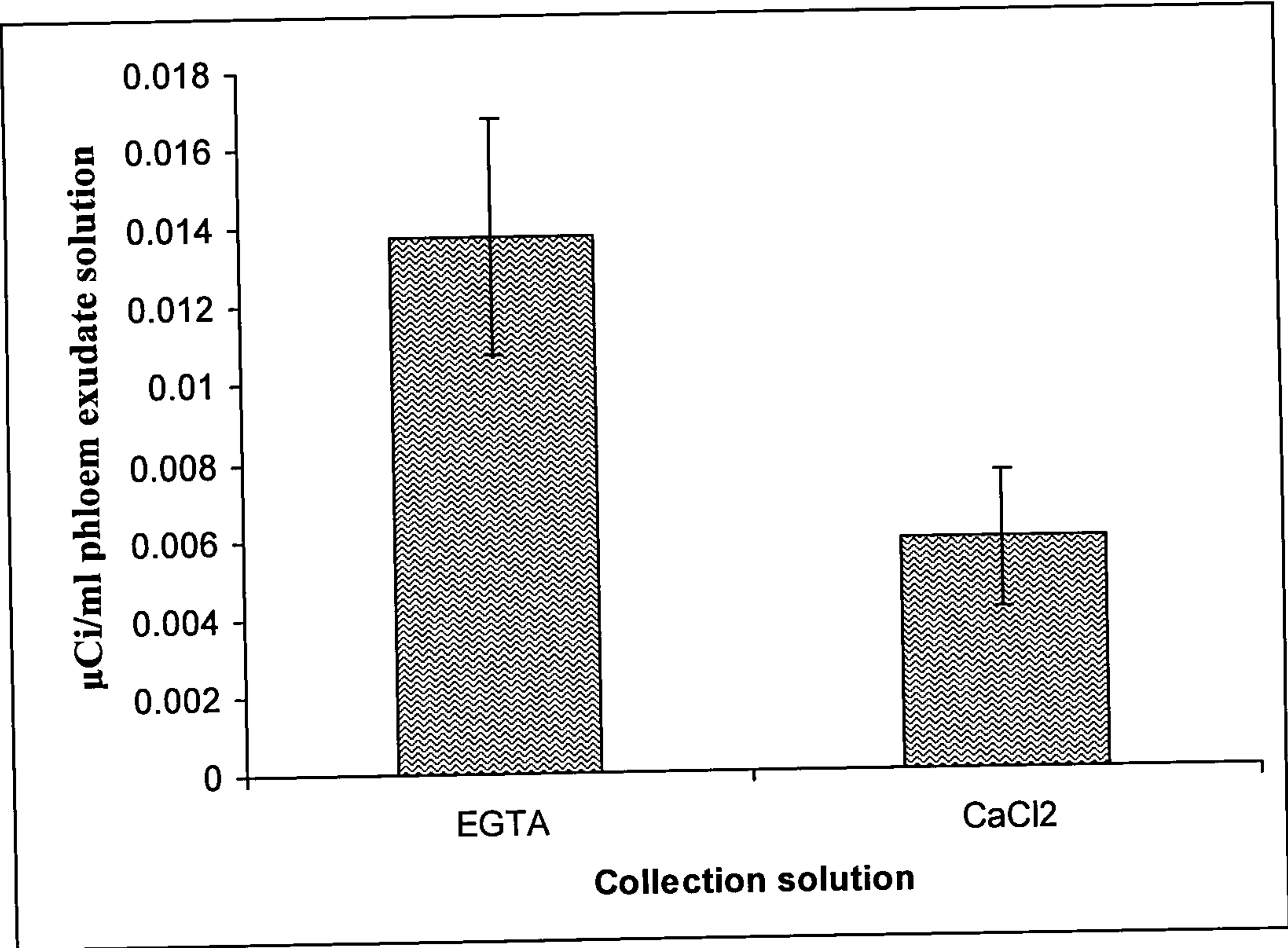
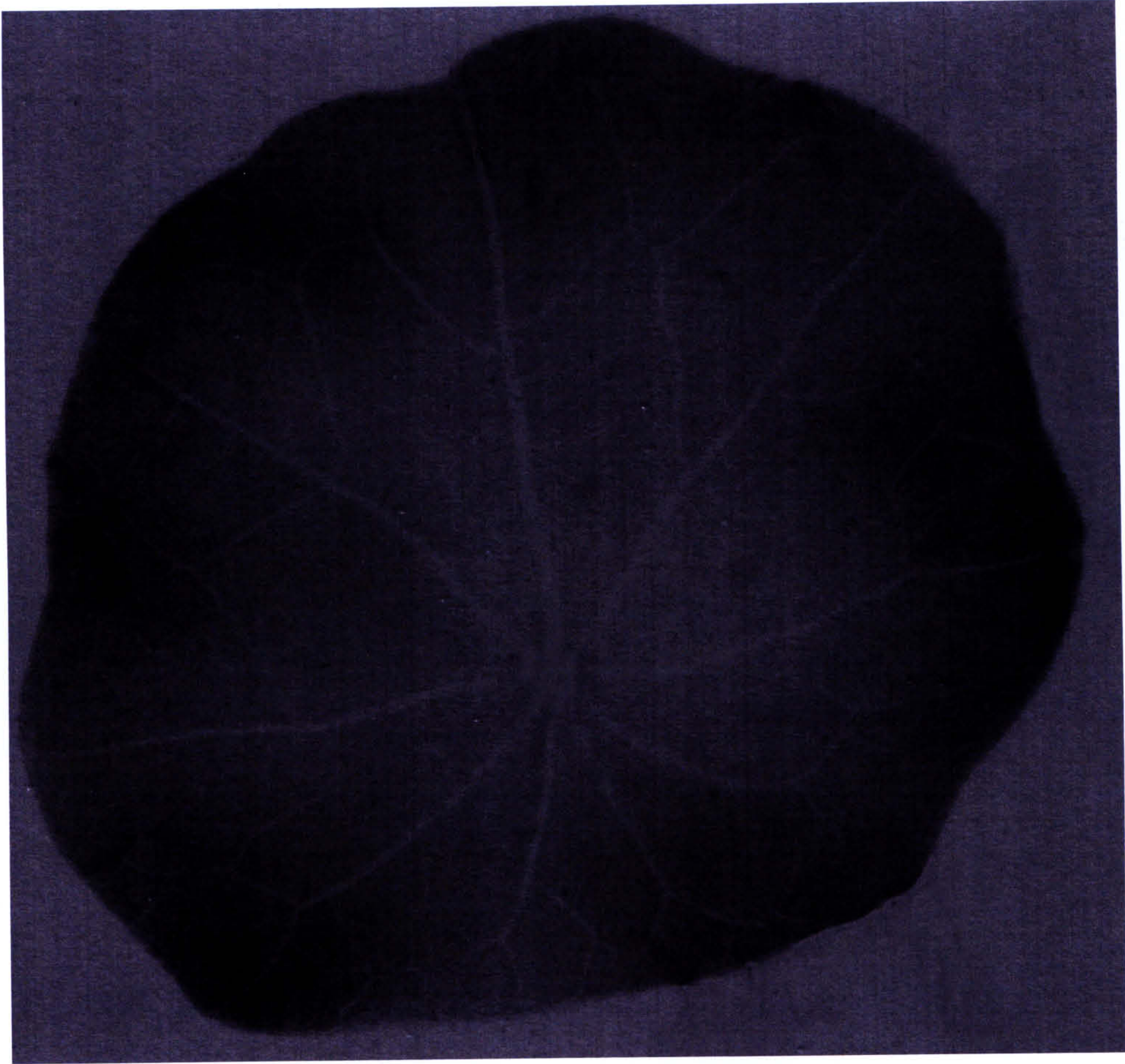


Figure 4.5.13. **The distribution of [<sup>3</sup>H]-dihydroazadirachtin within the leaf lamina.** Nasturtium plants were loaded with [<sup>3</sup>H]-dihydroazadirachtin. After loading, leaves were detached and the abaxial surface exposed to film. [<sup>3</sup>H]-dihydroazadirachtin was visualised upon developing the film. Darkened areas represent the presence of radioactivity. **(A)** Distribution of [<sup>3</sup>H]-dihydroazadirachtin throughout leaf. **(B)** Close-up section of autoradiograph.



(A)



(B)

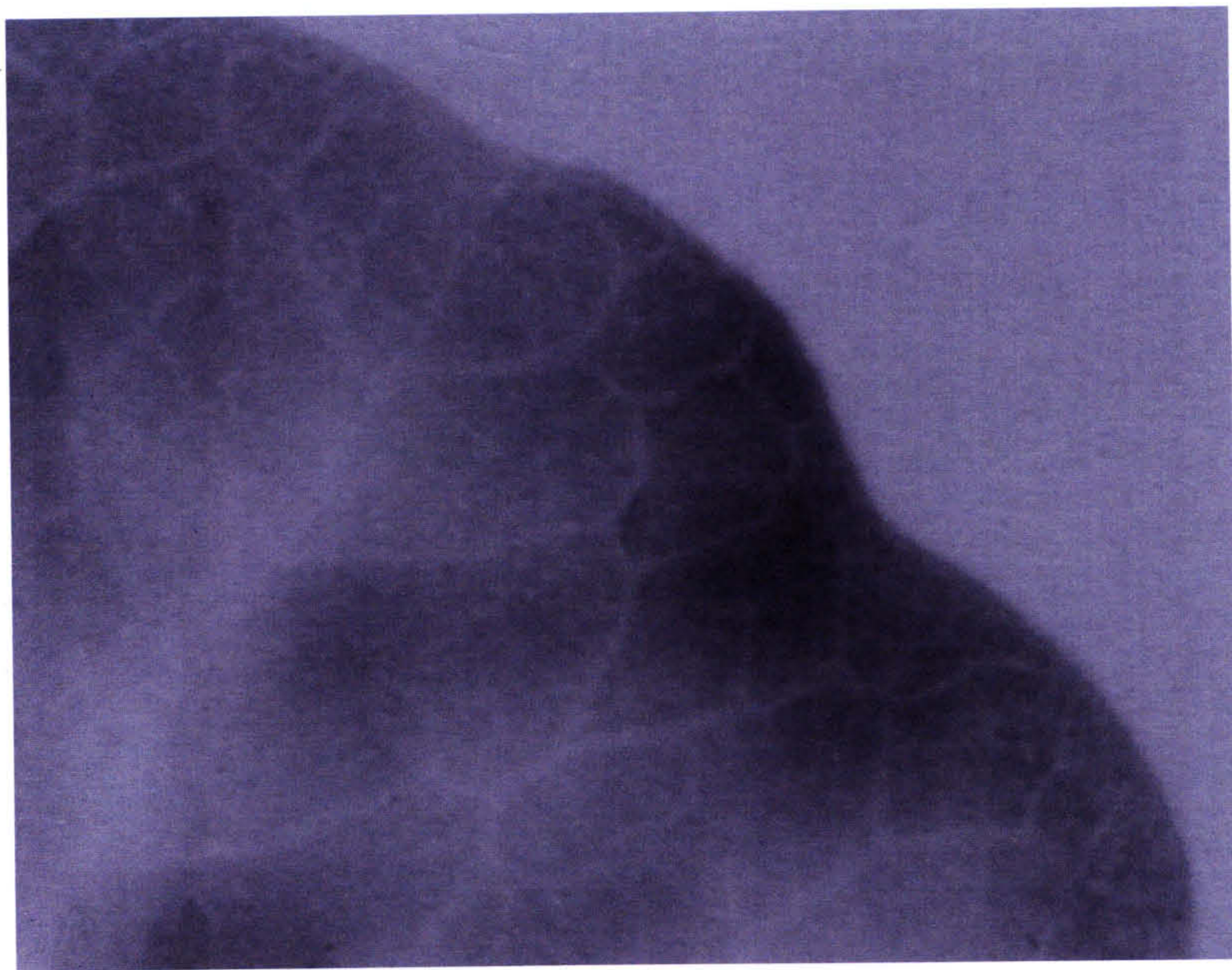
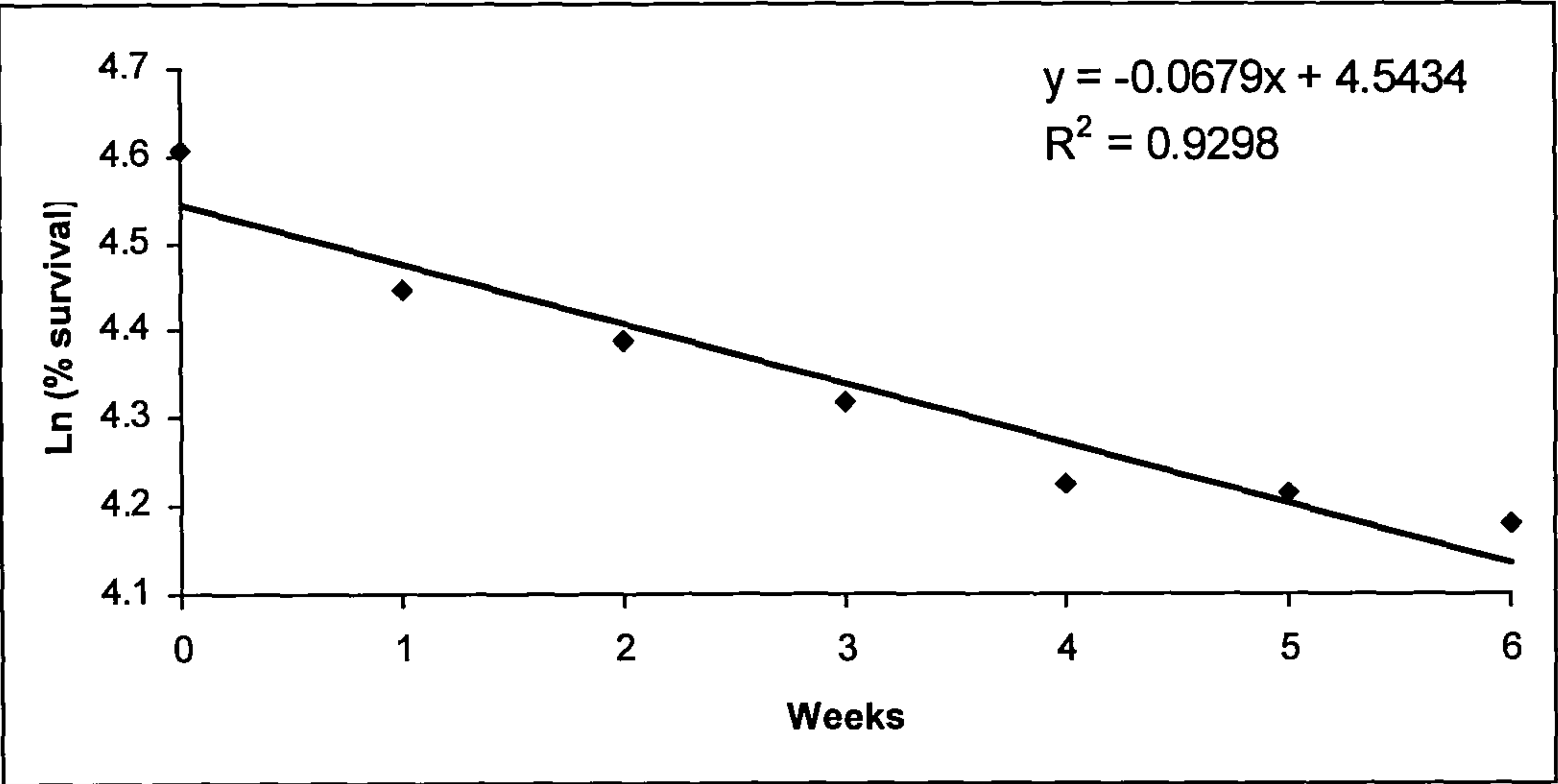




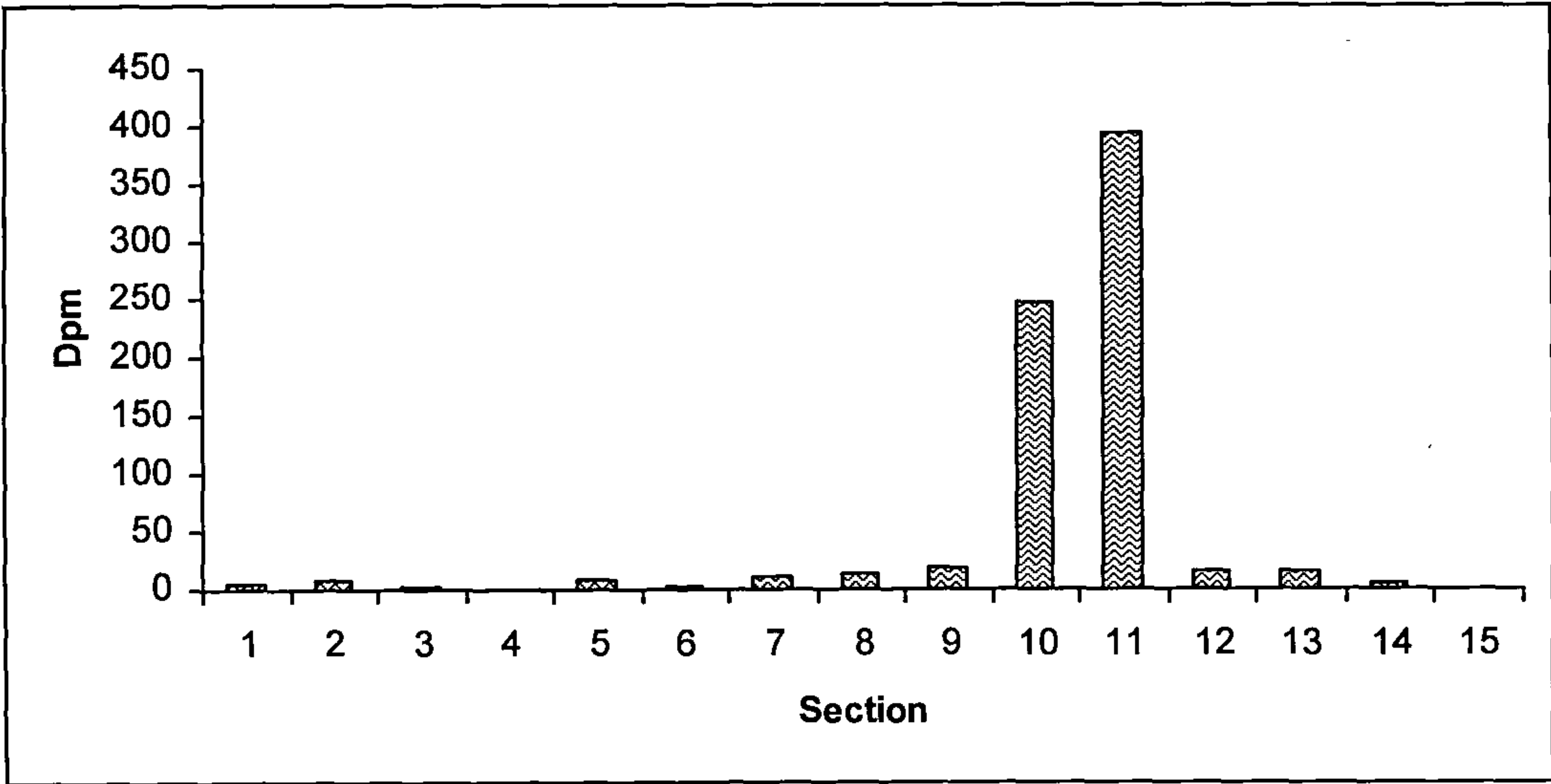
Figure 4.5.14. **Metabolism of [ $^3\text{H}$ ]-dihydroazadirachtin within plants.** Seven nasturtium plants were loaded with [ $^3\text{H}$ ]-dihydroazadirachtin. After loading, plants were transplanted into peat. At assessment points all leaves from a single plant were detached. [ $^3\text{H}$ ]-dihydroazadirachtin and breakdown products were extracted from leaf material into methanol. Aliquots were analysed by TLC (solvent system = methanol/ethyl acetate at 5:95). The TLC plate was cut into 15, 5mm sections. Increasing section number represents increasing distance from the point of origin (= section 1). Half-life was calculated from a plot of natural log (percentage of material remaining) against time. **(A)** Breakdown of [ $^3\text{H}$ ]-dihydroazadirachtin within plants (half-life: 9.3weeks). **(B)** Example of [ $^3\text{H}$ ]-dihydroazadirachtin standard following development by TLC. **(C)** Example of degraded [ $^3\text{H}$ ]-dihydroazadirachtin standard following development by TLC.



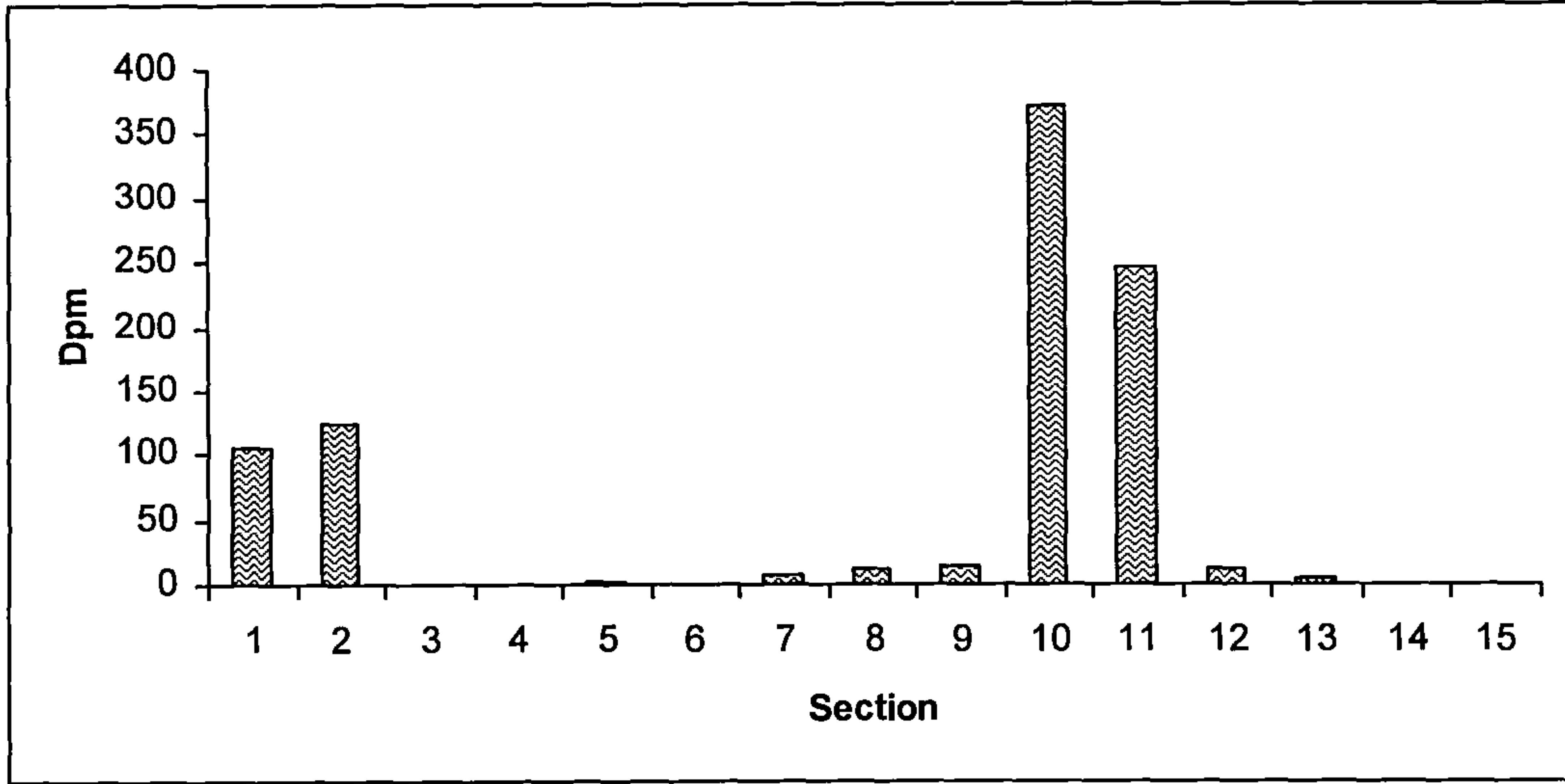
(A)



(B)



(C)



## **4.6. Discussion**

### **4.6.1. A comparison of azadirachtin with [ $^3\text{H}$ ]-dihydroazadirachtin**

The behaviour of pesticides within soils and plants has often been characterised using radiolabelled derivatives. For example, Cheah *et al.*, (1997) used  $^{14}\text{C}$ -labelled lindane to examine the partitioning characteristics of the herbicide within Malaysian agricultural soils. Similarly, Bromilow *et al.*, (1987) used  $^{14}\text{C}$ -labelled compounds during studies into the xylem and phloem behaviour of non-ionised insecticides within plants. Therefore, in order to follow and quantify the movement of azadirachtin within plant and soil systems, it was deemed appropriate to use a radiolabelled derivative of the limonoid. For this work it was determined that the most suitable option would be [22, 23,  $^3\text{H}_2$ ]-dihydroazadirachtin. This approach required that the tritiated derivative and the unaltered molecule were compared, to determine if both behaved similarly in the environment.

The two molecules were initially compared in terms of their *n*-octanol/water partition coefficients. As discussed previously within this chapter, this coefficient is used to predict the adsorption of pesticides to soil and therefore, can be used to give an estimate on soil mobility. The comparison revealed that azadirachtin and [ $^3\text{H}$ ]-dihydroazadirachtin partitioned in an almost identical manner between the two liquids. Both were observed to have greater lipid solubilities than aqueous solubilities. This study indicated that the two molecules differed by only 4% in their partitioning behaviours between the two systems. Consequently, this suggested that [ $^3\text{H}$ ]-

dihydroazadirachtin would be suitable in representing azadirachtin transport within the soil.

To compare the rate at which the two molecules are absorbed by roots and subsequently internally distributed to the leaves, a specific activity study was conducted. Nasturtium roots were immersed in an aqueous solution containing a mixture of azadirachtin and [ $^3\text{H}$ ]-dihydroazadirachtin for 24 hours. Following this period, the leaves of the plants were cut from their petioles and exhaustively extracted with methanol. The ratio of labelled to non-labelled molecule in these leaf extracts and in the initial loading solution was compared. This study revealed that the ratio remained 96% similar between the solution presented to the root and that within the leaf extract. However, [ $^3\text{H}$ ]-dihydroazadirachtin was taken-up by nasturtium plants marginally more readily as the ratio of labelled compared to non-labelled molecule increased within the leaf extract. Nevertheless, this research suggested that [ $^3\text{H}$ ]-dihydroazadirachtin could be regarded as an appropriate tracer in representing azadirachtin's root absorption and subsequent distribution within nasturtium plants.

This comparative work showed that the movement of azadirachtin from its point of application to the aerial plant regions, could be followed using a derivative of the limonoid that contained a tritiated label attached at the 22, 23 double bond. In addition, this radiolabelled tracer could also be used to investigate particular behavioural characteristics of azadirachtin within plants and soils (e.g. soil adsorption).

#### 4.6.2. Aqueous solubility of azadirachtin and [ $^3\text{H}$ ]-dihydroazadirachtin

Pesticides that are applied to soils must first dissolve before they can be transported within the water moving through the soil. Primarily, solutes dissolved in pore water will be transported by convective mechanisms, although diffusion and dispersion will also account for a significant amount of distribution. Consequently, the level of a compound's aqueous solubility will influence the extent of its transport by these processes (Gawlik *et al.*, 1997; Hartley & Graham-Bryce, 1980a and Leeds-Harrison, 1995).

Azadirachtin was shown to have a moderate aqueous solubility of 1.29g/l. This can be compared to systemic insecticides that are successfully applied to the soil for root-uptake by plants. For example aldicarb has an aqueous solubility of 4.93g/l, pirimicarb (3.0g/l), ethiofencarb (1.8g/l), imidacloprid (65mg/l) and disulfoton (25mg/l) (Tomlin, 1997). Therefore, this comparison shows that azadirachtin's solubility in water falls well within the typical range. This would suggest that the limonoid is sufficiently soluble for transport in soil water.

#### 4.6.3. Adsorption of azadirachtin to organic matter and clay minerals

Although pesticide transport within soils is mainly associated with water movement, the process has significant capacity of being modified by sorption mechanisms (Meyer-Windel & Lennartz, 1995). This influences the transport of solutes profoundly, because chemicals adsorbed on solid surfaces are effectively immobile



compared with flow and only that which is free in moving soil water is directly transported (Hartley & Graham-Bryce, 1980a).

The physical adsorption of pesticides to soil is often correlated with soil organic matter and can usually be well estimated from the lipophilicity of the pesticide (Nicholls, 1988). Consequently, the adsorption of azadirachtin to soil was predicted using the *n*-octanol/water partition coefficient ( $P_{ow}$ ).

Azadirachtin was calculated to have a  $\log P_{ow}$  of 0.85, which suggests that the limonoid was less than 10 times more lipid soluble than water soluble. Once again, this value can be compared to systemic insecticides that are successfully applied to the soil for root-uptake by plants. For example disulfoton ( $\log P_{ow} = 3.95$ ), ethiofencarb ( $\log P_{ow} = 2.04$ ), pirimicarb ( $\log P_{ow} = 1.7$ ), acetamiprid ( $\log P_{ow} = 0.80$ ), imidacloprid ( $\log P_{ow} = 0.57$ ), aldicarb ( $\log P_{ow} = 0.053$ ), oxamyl ( $\log P_{ow} = -0.44$ ) and methamidophos ( $\log P_{ow} = -0.80$ ) (Tomlin, 1997). This comparison shows that azadirachtin's  $\log P_{ow}$  is well within the typical range. Therefore, this comparison suggests that azadirachtin should move successfully from its point of application within the soil to the roots for uptake by plants.

However, to understand better the likely distribution of azadirachtin between the solid and liquid phases of soils, adsorption trials were conducted. These employed the use of [ $^3\text{H}$ ]-dihydroazadirachtin and assessed the extent to which clay minerals and organic matter would potentially remove azadirachtin from soil water. The sorbent materials tested were peat (containing an organic matter content of 94%, with little or

no clay) and four soils (containing a clay content that ranged from 1.2%-43%, with low amounts of organic matter).

The study revealed that peat was the only material that significantly sorbed [<sup>3</sup>H]-dihydroazadirachtin. The four soils were relatively non-sorbent in comparison, although it was observed that increasing clay mineral content correlated to increased adsorption throughout the different soil types. However, it should be noted that the increased adsorption might have also related to the organic matter content of each soil. This may have been additionally or alternatively responsible, as soils with increased clay content also exhibited slightly elevated levels of organic matter.

In this respect, Nicholls (1988) noted that the specific effects of clay minerals on the movement of pesticides in soils were difficult to isolate because clay content is weakly correlated with organic matter content in arable soils. Nevertheless, the percentage of [<sup>3</sup>H]-dihydroazadirachtin adsorbed by peat, in comparison to the four soils, suggests that organic matter is the main sorbent material and that clay minerals have little retention for the limonoid.

The findings of the adsorption study presented within this chapter, are in agreement with general observations published in the current literature. These have established that correlations between soil properties and the behaviour of un-ionised pesticides, such as adsorption, are nearly always stronger for organic matter than clay content (Nicholls, 1988).

The observation that azadirachtin is adsorbed principally by organic matter implies that applications to soils comprised primarily of clay, may result in considerable vertical displacement (i.e. leaching) of the limonoid. Subsequently, this may give rise to groundwater contamination or alternatively a loss of compound from zones of primary pesticide uptake (i.e. the rhizosphere).

However, the application of azadirachtin to soils of high organic matter content (e.g. when compost is included as an organic amendment) may give rise to contrasting distribution problems. Increased levels of adsorption may result in the reduced aqueous availability of the limonoid at root uptake sites, subsequently resulting in decreased concentrations absorbed by the plant.

The affinity of azadirachtin towards organic matter may have additional consequences regarding the potential behaviour of the limonoid within soils. Huang and Lee (2001) correlated the presence of dissolved organic matter (e.g. animal waste-derived effluents used as fertiliser) within soils to increased leaching potentials of the insecticide chlorpyrifos. This increase in mobility was associated to a reduction in adsorption by soil particles. Beck and Jones (1996) also investigated this aspect of pesticide soil behaviour and suggested that enhanced movement could occur because dissolved organic matter would serve as an additional partition/adsorption medium. Consequently, based on these observations, azadirachtin may also be susceptible to this form of enhanced transport within soils.

The adsorption of azadirachtin to organic matter may not only have consequences for the limonoid's mobility within soil. Basedow (2003) related azadirachtin's adsorption

by soil organic matter to the efficiency of insect control on *Vicia fabae* plants. This study correlated increasing soil organic matter content to extended periods of insect mortality. This effect was attributed to the observation that bound azadirachtin was being gradually released from organic particles. This in turn supplied the limonoid to the plant roots over an extended duration.

The sorption of azadirachtin to organic matter may also influence the persistence of the limonoid within the soil. Prolonged compound stability is often associated with adsorption of pesticides to soil because bound chemicals are less accessible to microorganisms that utilize exclusively or preferentially chemicals in solution. Therefore, following application, azadirachtin's persistence may be extended by the organic matter content of the soil concerned (Megharaj *et al.*, 1999).

However, Nicholls *et al.*, (2000) and Guo *et al.*, (2000) noted that two processes typically offset the influence of organic matter on pesticide persistence. Firstly, increasing organic matter content is often associated with an increased microbial biomass. Secondly, following adsorption, breakdown mediated by chemical reactions can be accelerated. Therefore, as a result of these processes, organic matter content may only play a limited role in influencing the stability of azadirachtin within soils.

#### 4.6.4. Desorption of azadirachtin from organic matter and clay minerals

Mobility studies that have investigated the movement of pesticides through soil have shown that the reversibility of adsorption has important consequences for transport within this media, particularly in the surface soil layers (Walker *et al.*, 1995).



Therefore, the desorption of [ $^3\text{H}$ ]-dihydroazadirachtin from peat and the four soils was investigated. These trials indicated that a large part of the initially fixed [ $^3\text{H}$ ]-dihydroazadirachtin was easily desorbable, evidently only being weakly bound to the support.

However, these trials also demonstrated that desorption from organic matter does not occur as readily as the process does from clay minerals. This was indicated by the low amount of tracer recovered from peat substrates in comparison to the clay containing soils. Consequently, the slow release of [ $^3\text{H}$ ]-dihydroazadirachtin from lipophilic adsorption sites would suggest that organic matter would further reduce the soil mobility of azadirachtin.

Therefore, the elution of azadirachtin through the soil profile will not only be slowed by processes of removal from transporting water (i.e. adsorption), but will also be further modified by the rate at which it is gradually returned back to this mobile phase (i.e. desorption into water).

#### 4.6.5. Sorption coefficients of azadirachtin

The adsorption of pesticides by soils has frequently been expressed using the soil sorption coefficient ( $K_d$ ) and the soil organic carbon sorption coefficient ( $K_{oc}$ ) (Walker *et al.*, 1995). Therefore, azadirachtin's mobility was further characterised using these coefficients that were derived from the adsorption studies of [ $^3\text{H}$ ]-dihydroazadirachtin.

The greater tendency of organic matter, rather than clay minerals, to bind [ $^3\text{H}$ ]-dihydroazadirachtin was reflected in the soil sorption coefficients. The soil sorption coefficient of [ $^3\text{H}$ ]-dihydroazadirachtin towards peat was approximately nine times greater than the highest value derived for any soil type containing a high clay content

However, in describing the transport of pesticides through soils, the soil organic carbon sorption coefficient is more commonly used. The  $K_{oc}$  value assumes that all adsorption is related to the organic carbon fraction of the soil. In addition, the approach makes several theoretical assumptions regarding the formation of an adsorption equilibrium and that this process is completely reversible (Leake & Gatzweiler, 1995). Nevertheless, McCall *et al.*, (1980) developed a mobility classification scheme based on calculated  $K_{oc}$  values for pesticides. This system has been commonly used to identify pesticides either as immobile ( $K_{oc} > 5000$ ), slight mobility ( $K_{oc}$  2000-2500), low mobility ( $K_{oc}$  500-2000), medium mobility ( $K_{oc}$  150-500), high mobility ( $K_{oc}$  50-150) and very high mobility ( $K_{oc}$  0-50). Consequently, despite considerable adsorption to, and slow desorption from organic matter, [ $^3\text{H}$ ]-dihydroazadirachtin can still be regarded as very highly mobile within the soil environment as all  $K_{oc}$  values were below 50.

[ $^3\text{H}$ ]-dihydroazadirachtin had a mean  $\log K_{oc}$  value of 1.51. This value can be compared to those of existing soil applied, systemic insecticides. For example phosphamidon ( $\log K_{oc} = 2.26$ ), pirimicarb ( $\log K_{oc} = 1.90$ ), aldicarb ( $\log K_{oc} = 1.50$ ), methamidophos ( $\log K_{oc} = 1.27$ ) and oxamyl ( $\log K_{oc} = 1.00$ ) (Gramatica & Guardo, 2002). Azadirachtin's  $\log K_{oc}$  clearly falls within this range. Therefore, this comparison would suggest that azadirachtin's partitioning behaviour between

stationary organic carbon and the mobile aqueous phase is appropriate for soil application.

#### 4.6.6. Persistence of azadirachtin within soils

The successful use of azadirachtin as a soil-applied insecticide will require that the limonoid exhibits an adequate level of persistence. Persistence describes how long a molecule or its metabolites remain untransformed and not dissipated in the environment (Leake *et al.*, 1995). The term metabolite in this context refers to all reaction or breakdown products of an active substance, which are formed in the environment after application (SANCO, 2003).

The dissipation of organic agricultural chemicals in soils results from a combination of mechanisms, such as chemical hydrolysis, microbial breakdown, photochemical degradation, volatilisation, leaching and surface run-off. In particular, chemical hydrolysis and microbial breakdown are regarded as the most important pesticide dissipation processes (Morrica *et al.*, 2001 and Walker & Allen, 1984).

The breakdown of azadirachtin within the soil was studied using methods that have been employed by numerous authors, for example Bromilow *et al.*, 1999; European Commission, 2000 and Morrica *et al.*, 2001. Within a range of freshly collected soils at 20°C, and under aerobic conditions, azadirachtin was shown to have a relatively short half-life ranging from 1.06 days to 11.9 days.

The breakdown studies showed evidence of biphasic degradation within all soils types studied. Leake *et al.*, (1995) observed that the transformation or dissipation of pesticides in soils is often not linear. Within the four soil types, there would appear to be an initial slow period of breakdown followed by a more rapid phase. Consequently, if this is accounted for, the half-life of azadirachtin was shown to change over time. Within the sandy loam soil, the half-life of azadirachtin reduced from an initial 11.9 days to 2.70 days over the nine-day observation period. The half-life of azadirachtin within the sandy clay loam soil was initially 6.17 days, but this decreased to 1.06 days over the nine-day observation period. Similarly, in the clay soil azadirachtin's half-life fell from an initial 3.74 days to 1.43 days by the end of the observation period. The same characteristic breakdown of azadirachtin was also shown in the silty clay soil, where the half-life of the limonoid dropped from 5.27 days to 2.06 days. This initial lag in breakdown has been recorded in a number of studies investigating the dissipation of pesticides within the soil, for example Cox *et al.*, (1996); Martins & Mermoud (1998) and Nicholls *et al.*, (2000).

Nicholls *et al.*, (2000) observed that this behaviour was a common feature of a batch culture system (as used within this chapter, see section 4.4.10.). This author also proposed that biphasic breakdown could be associated with microbial growth occurring following the addition of pesticide to soil. Das & Mukherjee (2000) studied this effect and made further conclusions regarding how the response could occur. From this research it was noted that the addition of HCH, phorate, carbofuran or fenvalerate to the soil, increased the populations of certain microorganisms (e.g. *Bacillus*, *Proteus*, *Corynebacterium*, *Streptomyces*, *Fusarium*, *Trichoderma* and *Rhizopus*). Simultaneously, the same insecticides exerted deleterious effects on other



populations (e.g. *Pseudomonas*, *Staphylococcus*, *Nocardia*, *Micromonospora* and *Aspergillus*). This work demonstrated that the death of a portion of the soil microbial biomass increased the available nitrogen and phosphorus in the soil. Those microorganisms not sensitive to the insecticides were then able to utilize the nutrients with a resultant increase in their population size.

Therefore, this effect may explain why biphasic breakdown was observed to have occurred in some soils (i.e. sandy loam and sandy clay loam) but not in others (i.e. clay and silty clay). The non-linear dissipation of azadirachtin may have occurred only in soils in which the microbial biomass was composed of both susceptible and non-susceptible populations.

The above effect may suggest that microbial activity is primarily responsible for the degradation of azadirachtin within the soils studied, rather than chemical processes. However, it is possible to estimate breakdown associated with chemical mechanisms, such as aqueous hydrolysis. Figure 4.5.1. shows the pH of water within each of the soils studied. Clay and silty clay had a pH (water) of 7.5, sandy clay loam a pH (water) of 6.8 and sandy loam a pH (water) of 7. Therefore, these values can be used to estimate the extent of azadirachtin's aqueous hydrolysis (see figure 4.5.4. and section 4.6.8.), during the nine-day study period. Within the clay soil, 94% of the applied azadirachtin was degraded during the nine-day observation period. Of this, approximately 20% can be associated with aqueous hydrolysis. Azadirachtin applied to silty clay soil was degraded by 90% during the study. Approximately 20% of this can be associated with breakdown due to aqueous hydrolysis. Within the sandy clay loam soil, 100% of the applied azadirachtin was degraded after seven days. Over this

period, aqueous hydrolysis would have accounted for 8% of the total breakdown. Finally, azadirachtin applied to the sandy loam soil was degraded by 70% during the nine-day study period. Approximately 20% of this breakdown can be associated with degradation due to aqueous hydrolysis. These comparisons would suggest therefore, that microbial activity is primarily responsible for azadirachtin's breakdown in soils, although chemical mechanisms, which include aqueous hydrolysis will also account for a considerable breakdown.

#### 4.6.7. Identification of the azadirachtin metabolites in soil

Following exhaustive extraction of soils to remove azadirachtin and its breakdown products, HPLC analysis revealed that the limonoid was broken-down into a single, more polar metabolite. This material was identified by nuclear magnetic resonance (kindly performed by Professor Joe Conolly, Department of Organic Chemistry, University of Glasgow) as 3-deacetylazadirachtin (result not presented).

Chromatographic analysis of extracts also indicted that there was no apparent breakdown of the deacetylated metabolite within the soil throughout the duration of the nine-day study period. This was suggested as all eluants could be identified as 3-deacetylazadirachtin, soil constituents present in blank references, or components belonging to the azadirachtin standard.

The degradation of azadirachtin into this single metabolite has two important consequences. Firstly, 3-deacetylazadirachtin has been shown to exhibit notable biological activity against insects, for example in the *Epilachna varivestis* bioassay

(Kraus, 2002). Consequently, there is a possibility that even though the parent molecule (azadirachtin) is rapidly degraded within soils, the metabolite may exert an insecticidal effect, which is sufficient for plant protection purposes. However, the second consequence is from a regulatory perspective. Breakdown products, which account for more than 10% of the amount of active substance added to soil, and which exhibit comparable biological activity (i.e. over 50%) must be considered relevant and be subjected to the same regulatory controls as the parent (SANCO, 2003). Therefore, this would greatly increase the cost and complexity of registering an azadirachtin insecticidal formulation within the European Union.

There has been limited comparable research that has studied the dissipation of azadirachtin within the soil. Notably, Stark & Walter (1995) investigated the effects of temperature and microbial activity on the soil persistence of azadirachtin-A within Margosan-O formulations. This research showed that the DT<sub>50</sub> for the limonoid was 43.9 days and 19.8 days for soil maintained at 15°C and 25°C respectively. These results are considerably longer than the estimations determined in this work. This discrepancy may be explained by comparing organic matter contents of soils used in each trial. Those employed by Stark & Walter were considerably lower in organic matter content. Therefore, it can be predicted that the corresponding microbial biomass and subsequent total activity would have been reduced, resulting in decreased azadirachtin degradation rates. In addition, it is also possible that the formulants within the Margosan-O formulation were having a deleterious influence on microbial activity.

In contrast to Stark and Walter, the research presented within this chapter appears to be in agreement with unpublished findings by Trifolio-M GmbH (Wetzlar, Germany). This research investigated the persistence of the azadirachtin-based formulation NeemAzal within three soil types. Azadirachtin was observed to have a half-life that varied from 1.9 days to 3.8 days. In addition, this work also showed, via HPLC-MS, that the acetyl moiety was also cleaved from the parent as a result of microbial activity. The resulting metabolite was subsequently identified as 3-deacetylazadirachtin-A.

#### 4.6.8. Aqueous Hydrolysis of [ $^3\text{H}$ ]-dihydroazadirachtin

In addition to microbial breakdown, chemical hydrolysis is regarded as a second important process that will be involved in the dissipation of pesticides within soils. Therefore, the hydrolytic stability of azadirachtin in buffered distilled water was investigated using [ $^3\text{H}$ ]-dihydroazadirachtin.

The results suggest that the persistence of [ $^3\text{H}$ ]-dihydroazadirachtin was pH sensitive, with half-lives ranging from several hours under basic conditions to over fifty days under acidic conditions. The route of hydrolytic breakdown was not determined by this work.

The findings of this study are in agreement with Jarvis *et al.*, (1998) who investigated the stability of azadirachtin within aqueous and organic solvents, with a view to improving the conditions of isolation, storage and use of the limonoid. The work conducted by Jarvis *et al.*, also showed that hydrolytic breakdown occurred most



rapidly when azadirachtin was dissolved in alkaline solutions. Under mildly acidic conditions, the limonoid was considerably more stable. However, at extreme acid conditions (i.e. below pH 4) it was observed that the limonoid was once again rapidly hydrolysed. No attempt was made during these studies to identify azadirachtin's degradation route.

The pH of soil has been described as a factor that is involved in determining degradation rates of pesticides (Walker & Allen, 1984). Consequently, [<sup>3</sup>H]-dihydroazadirachtin's pH sensitive, hydrolytic breakdown may have important consequences for soil applications using preparations that contain azadirachtin.

Soils have considerable pH buffering capacity and subsequently, water within soils can have a broad pH range (White, 1979). Generally, the pH of soil water ranges from mildly acidic (pH 4) to mildly alkaline (pH 8) conditions. However, it has been determined for most plants that the optimal pH range for nutrient availability and hence growth is closer to neutral (i.e. pH 6.5 to pH 7.2) (Garden Gate, 2003). Nevertheless, the aqueous hydrolysis investigation would suggest that applications of azadirachtin to soils must account for soil water pH, as this may promote accelerated active substance breakdown.

An important consequence of azadirachtin's hydrolytic stability under acidic conditions is that degradation rates may be reduced by the addition of organic matter (e.g. compost) to soil. Worrall *et al.*, (2001) noted that organic matter tends to have an acidic pH and concluded therefore, that organic amendments could confer a degree of protection to pH-sensitive pesticides within the soil.

#### 4.6.9. Root uptake and systemic distribution of [ $^3\text{H}$ ]-dihydroazadirachtin from soil applied granules (part 1)

The effective distribution of a granular formulation is mechanically more difficult to accomplish than distributing a liquid in the form of a spray. Often, the broadcast application of granular formulations leads to non-uniform distribution of the active substance within soils (Furmidge, 1984). This in turn influences the relative rate at which a released pesticide is supplied to the rhizosphere and subsequently absorbed by roots (Basedow, 2003 and Hartley and Graham-Bryce, 1980b).

Therefore, two application methods were investigated to compare the effect of granule placement on the quantities of [ $^3\text{H}$ ]-dihydroazadirachtin released by the formulation and on amounts accumulated by nasturtium plants over time. The quantities released by the granules into the compost water were the same irrespective of granule placement. However, the amounts accumulated by the plants were initially affected by the position of the granules in the compost, where concentrations in plant tissues at 14 days after application were considerably lower when granules were applied to the compost-surface only.

These results suggest that granule placement does effect that rate at which [ $^3\text{H}$ ]-dihydroazadirachtin is accumulated by plants, but not the speed at which it is released from the formulation. The likely explanation for the effect on accumulation rates, relates to granule position relative to the sites of compound uptake on the root (i.e. root apices). Consequently, compared to a homogeneous granule distribution, the limonoid released from granules on the compost surface must travel further prior to

root absorption. Therefore, this process will produce a lag phase during which the released azadirachtin is percolating down through the compost profile. Simultaneously, the concentration of the limonoid at the root zone will be relatively low in comparison to the total amounts released into the soil at higher positions. In comparison, granules mixed throughout the compost will have a more uniform distribution, with some [ $^3\text{H}$ ]-dihydroazadirachtin release occurring directly into the rhizosphere. Therefore, this will decrease the distance that must be travelled prior to root absorption, subsequently reducing the duration of the lag phase to less than 14 days, where it would not have been recorded.

This effect may have important consequences for insect pest control. The results indicate that broadcast granule applications may exhibit a period of reduced plant protection when compared to that offered by formulations uniformly distributed within the soil. This period will relate to the time taken for the released azadirachtin to initially move from the granule to the root uptake zone.

The uptake study also revealed that if granules were mixed uniformly throughout the compost, the concentration of [ $^3\text{H}$ ]-dihydroazadirachtin within plant tissues remained relatively constant between the 14-day and 28-day assessments. Several authors (for example Inoue *et al.*, 1998; Raveton *et al.*, 1997 and Trapp, 2000) have also made this observation during research into the root uptake of pesticides under hydroponic conditions. These studies have associated this effect with an equilibrium being established between the external solution that is supplied to the root and the internal plant solution.

From these investigations it has been suggested that plants can go through an initial rapid phase of physical uptake, followed by a very slow accumulation of material. After this period, both the internal aqueous and non-aqueous phases may approach some form of equilibrium with each other and with the external solution. The precise point at which this equilibrium occurs at being dependant on the metabolic processes of the plant species concerned and the partitioning characteristics (i.e. between the lipid and aqueous phases) of the pesticide being studied (Moody *et al.*, 1970 and Sicbaldi *et al.*, 1997).

The equilibrium that is established may potentially reduce the application efficiencies of neem formulations applied to the soil. This might occur because, despite there being azadirachtin available for root uptake, the proportion absorbed by roots may be limited by the concentration within the plant relative to that within the soil solution. As a consequence, processes such as biological and chemical breakdown will dissipate the azadirachtin that remains free in the soil water.

During these investigations it was not possible to extract bound residues from peat with a high efficiency. Consequently, an assessment of concentrations adsorbed to organic matter is not provided.

#### 4.6.10. Root uptake and systemic distribution of [<sup>3</sup>H]-dihydroazadirachtin from soil applied granules (part 2)

Following root uptake by nasturtium plants, the internal distribution of [<sup>3</sup>H]-dihydroazadirachtin via the vascular system was investigated. This revealed that a



concentration gradient existed within the plant, where older leaves occupying lower stem positions contained higher concentrations of the tracer. This effect can be explained by the observation that older leaves had longer to accumulate [ $^3\text{H}$ ]-dihydroazadirachtin. When granules were applied (two weeks after germination), only two leaves per plant had developed.

From this investigation, some basic observations can be made regarding the total amount of [ $^3\text{H}$ ]-dihydroazadirachtin absorbed by plants over time. The quantities within leaves suggest that even though concentrations of [ $^3\text{H}$ ]-dihydroazadirachtin within nasturtium plants remained relatively constant between the 14-day and 28-day assessments, the total amount absorbed by the plants would have increased in line with increasing plant size.

There are few comparable works that have investigated the distribution of azadirachtin within plants following root absorption. Sundaram (1996) studied accumulation characteristics within young spruce trees. This research also noted that azadirachtin was readily absorbed by roots and transported via the vascular system. Supporting the results presented within this chapter, Sundaram showed that after an initial accumulation, which lasted 5 days, the concentration of the limonoid within various parts of the tree remained relatively constant. Additionally, Basedow (2003) recorded a similar effect during investigations into the uptake of azadirachtin by *Vicia fabae* plants. During these studies it was also observed that maximum concentrations of the limonoid within leaves was reached 5 days after application.

#### 4.6.11. The xylem and phloem mobility of azadirachtin

The mobility of azadirachtin within the apoplasm was directly assessed. This was achieved by collecting xylem exudate from de-topped nasturtium plants that had absorbed [ $^3\text{H}$ ]-dihydroazadirachtin at the roots. Following this collection, liquid scintillation counting revealed that significant quantities of radioactivity were present within the exudate. This confirmed the movement of the tracer within xylem vessels.

Azadirachtin's mobility within the symplasm was determined based on methodology and observations made by King and Zeevaart (1974) who developed techniques to distinguish between the phloem and xylem exudate released from cut petioles. This research demonstrated that phloem exudation could be enhanced using petiole-bathing solutions that contained various chelating agents (e.g. EGTA). In addition, it was observed that bathing solutions containing  $\text{CaCl}_2$  could be used to prevent exudation from these vessels. This study explained this effect and established that chelating agents were effective in sequestering the divalent cations (e.g.  $\text{Ca}^{2+}$ ) involved in callose formation. In contrast, by supplying these cations using a  $\text{CaCl}_2$  solution, the opposite effect could be achieved and the development of callose could be promoted. Callose forms in response to phloem vessel damage and is implicated in the physical blockage of sieve tubes. Therefore, this biological response can be used as the basis to distinguish between phloem and xylem exudate, as xylem vessels are not blocked by this mechanism.

Therefore, nasturtium plants were loaded with [ $^3\text{H}$ ]-dihydroazadirachtin under hydroponic conditions. Petioles were subsequently cut and the exudate collected into

one of two bathing solutions (either EGTA or  $\text{CaCl}_2$ ). Based on the requirements for callose formation (i.e.  $\text{Ca}^{2+}$ ), any radioactivity recovered into  $\text{CaCl}_2$  solutions must have originated from xylem vessels, as phloem vessels would have been blocked. Alternatively, quantities recovered into EGTA solutions would have originated from both xylem and phloem vessels, as sieve tubes would have remained in an unblocked functional state (as observed by King and Zeevaart). Consequently, the presence of [ $^3\text{H}$ ]-dihydroazadirachtin within phloem vessels could be calculated by subtracting the total radioactivity within the  $\text{CaCl}_2$  collection solution from the amounts within the EGTA collection solution.

The results showed that following sieve tube blockage, significant quantities of radioactivity were collected into petiole bathing solutions containing  $\text{CaCl}_2$ . This quantity represented the amount of tracer that was returning from xylem vessels into solution. The radioactivity collected within petiole bathing solutions containing EGTA represented the combined quantities from xylem and phloem vessels and was significantly higher compared to  $\text{CaCl}_2$  solutions. The calculated difference suggested that [ $^3\text{H}$ ]-dihydroazadirachtin was present within phloem exudate.

These results suggest that within nasturtium plants azadirachtin is ambimobile and thus capable of transport within both xylem and phloem vessels. This ability is regarded as highly desirable in a plant protection product as it offers the possibility of simultaneously controlling general defoliators (e.g. species belonging to Orthoptera, Lepidoptera, Diptera, Coleoptera and Hymenoptera), as well as sucking pests that feed exclusively on the contents of sieve elements (e.g. species belonging to the orders Hemiptera (Heteroptera and Homoptera) and Thysanoptera).

The mobility of azadirachtin within both phloem and xylem vessels may also influence the stability of the limonoid within plants. Hartley & Graham-Bryce (1980b) provided evidence to show that xylem fluid is relatively acidic (pH 5-6), whereas phloem fluid is mildly alkaline (pH 7-8). Azadirachtin's pH sensitive, hydrolytic breakdown (presented previously within this chapter) could therefore be responsible for accelerated degradation of the limonoid during transport within phloem vessels.

#### 4.6.12. The distribution of [ $^3\text{H}$ ]-dihydroazadirachtin within the leaf lamina

Leaves loaded with [ $^3\text{H}$ ]-dihydroazadirachtin were subjected to autoradiography. This examined the likely lateral distribution of azadirachtin throughout the leaf lamina following movement within the transpiration stream. Autoradiographs indicated that [ $^3\text{H}$ ]-dihydroazadirachtin was more or less evenly distributed throughout the leaf tissue of nasturtium plants, although there did appear to be some increased accumulation at leaf edges. The autoradiographs also suggested that the tracer did not accumulate within the leaf veins, as evidence by reduced darkening of film along these vessels when compared to interveinal areas. This effect, may help explain observations made by Blaney & Simmonds (2002) who presented evidence to show that neem derivatives are generally more potent as antifeedants against phytophagous chewing insects, such as locusts and lepidopterous larvae, than towards sap feeding insects, such as aphids. Clearly, as azadirachtin is not accumulated within leaf veins, insect pests that feed for example, exclusively on the contents of the phloem vessels, will consume far lower doses when compared to defoliators. However, this result should be interpreted with caution. This is because the reduced darkening along veins,



as well as the increased darkening at leaf edges, may alternatively be the result of a variable thickness in the cuticular membrane (including cuticular and epicuticular waxes), as observed by Kirkwood (1999). Thicker wax layers would have reduced the level of exposure and therefore, darkness of the film.

Nevertheless, if azadirachtin is more or less distributed uniformly throughout the leaf, this would suggest that the corresponding level of insect protection should also be nearly uniform throughout the tissue. This may be particularly important when considering the primary antifeedant effect of azadirachtin against phytophagous chewing insects (see Chapter 1). This insecticidal action relies on behavioural avoidance being initiated following the perception of the limonoid by chemoreceptors on the insect's mouthparts and feet. Consequently, being uniformly distributed throughout the leaf tissue may help to ensure that insects do not settle or preferentially feed on any localised regions of protected plants that contain lower amounts of azadirachtin.

#### 4.6.13. The metabolism of [ $^3\text{H}$ ]-dihydroazadirachtin within plants

Higher plants have an extensive ability to metabolise pesticides. This occurs through phase I (oxidative, hydrolytic, reductive or group transfer reactions) and/or phase II (conjugation with glucose, glucuronic acid, phosphate or amino acids) steps (Wilkinson, 1984; Cole, 1994 and Commission of the European Communities, 1997). Therefore, the stability of azadirachtin within plants was investigated using [ $^3\text{H}$ ]-dihydroazadirachtin. The radiolabelled tracer was required for this work as initial investigations revealed that HPLC analysis would fail to resolve polar breakdown

peaks from the large amount of interference caused by chlorophyll within leaf extracts.

The study revealed that following root absorption [ $^3\text{H}$ ]-dihydroazadirachtin was stable, having a half-life of approximately 9 weeks. Examination of extracts by TLC also showed that the tracer was degraded into a single metabolite of increased polarity. The metabolite was not identified at this time because of a lack of analytical reference standards for comparison. However, based on the soil metabolism study, a possibility is that the metabolite is 3-deacetylazadirachtin. If this assumption were correct, then it would suggest that the route of azadirachtin breakdown is similar in plants and in soils. Alternatively, the increased polarity of the [ $^3\text{H}$ ]-dihydroazadirachtin metabolite may indicate that conjugation with a natural plant constituent (e.g. glucose) has occurred. This metabolic process can occur following phase I metabolism, or alternatively with the unaltered molecule, particularly where hydroxyl, phenolic and carboxylic acid functional groups are present (Cole, 1994 and Edwards *et al.*, 1982).

The apparent stability of the tracer within plant tissues has important consequences for plant protection using azadirachtin. This result would suggest that following root absorption, the environmental persistence of the limonoid will be extended, especially when compared to predicted dissipation times within soils and water. Consequently, the efficiency of azadirachtin as a plant protection agent may be improved.

However, because the limonoid is extremely persistent within plants, there may be problems regarding the accumulation of potentially phytotoxic levels within tissues.

This may be a valid concern as azadirachtin has been shown to exhibit strong phytotoxic effects towards numerous types of plants, causing in such instances symptoms including yield loss and necrosis (Schmutterer, 1995).

There is no comparable research with azadirachtin that can be used to aid in the interpretation of these results. However, the increased stability of [<sup>3</sup>H]-dihydroazadirachtin within plants may be the result of the tracer associating with insoluble cellular constituents (e.g. lignin and/or polysaccharides) in a poorly understood process termed phase III metabolism that is also referred to as compartmentation (Cole, 1994). As a consequence, like soil adsorption, this may confer a degree of protection from metabolically driven dissipation processes.

## Chapter 5

### Final Discussion and Conclusions



## **5.1. Final discussion**

### **5.1.1. Project overview**

The objective of this project was to assess the suitability of azadirachtin as an insecticide that is applied to the soil, using a granular formulation, for root uptake and subsequent systemic plant protection. To achieve this goal a number of studies were undertaken. These are briefly reviewed in the following sections.

## **5.2. Chapter 2 overview – Azadirachtin isolation and quantitative estimation of neem seed extracts**

### **5.2.1. Isolation of azadirachtin**

The isolation of azadirachtin is considered to be difficult. This is because neem seed kernels contain large quantities of oil and also because of the structural similarity of azadirachtin and its related limonoids (Turner *et al.*, 1987 and Yamasaki *et al.*, 1986). Purification procedures require the application of a number of techniques, although central to these is high performance liquid chromatography. As a consequence, the isolation of pure azadirachtin is characteristically expensive, time consuming and of low yield (Hein & Hummel, 2000 and Thejavathi *et al.*, 1995). Therefore, methods such as multilayer countercurrent chromatography and supercritical fluid extraction have been investigated as alternatives. However, these often suffer from the same disadvantages relating to speed and costs (Ambrosino *et al.*, 1999; Jarvis *et al.*, 1999 and Lou *et al.*, 1999).

As a result, the second chapter of this project aimed at addressing this point by developing a method, to rapidly isolate large quantities of azadirachtin-A. This material would be required later for chemical and biochemical studies. The purification process developed was based on reversed-phase flash chromatography and could achieve a return of 350mg azadirachtin-A (95% pure) from 4g starting material (35% pure) within a period of 2 hours. Compared to purification based on HPLC, the developed method offered speed and yield advantages, although it was inferior terms of the maximum purity of azadirachtin that could be isolated. Other practical advantages of the method, related to the use of non-flammable mixtures of methanol and water to make-up the mobile phase. Typically, solvent mixtures of ethyl acetate and petroleum ether are employed.

The objective of this part of Chapter 2 was achieved, however, the methodology may benefit from further development. It was observed during analytical HPLC that solutes could be separated more efficiently if the mobile phase was modified to contain a mixture of acetonitrile: water, rather than a solvent system of methanol: water. Consequently, if this is applied to purification based on flash chromatography, this modification may be a way to obtain higher purity azadirachtin, without making trade-offs regarding time and quantities isolated.

### 5.2.2. Quantitative estimation of neem seed extracts

Azadirachtin is quantified using relatively few methods. Typically, these are based on chromatography and suffer disadvantages relating to speed and specificity (Warthen *et al.*, 1984). Consequently, this has promoted research into alternative approaches

that can overcome these deficiencies. In particular, Dai *et al.*, (1999 & 2001b) has developed a method that involves adding azadirachtin to acidified vanillin solutions to produce coloured mixtures. The absorbance of these solutions, at a specific wavelength, relating directly to azadirachtin content. As a result, Dai *et al.*, has suggested that this colorimetric procedure offers the possibility of a technique that is both rapid and specific for azadirachtin quantification.

Therefore, the second aim of Chapter 2 was to estimate azadirachtin and related limonoids using this procedure and to compare these values with assessments based on HPLC. This comparison would indicate the suitability of the assay in rapidly quantifying azadirachtin within crude neem seed extracts and during environmental fate studies conducted at later points in the project. The study revealed that the method was generally unsuitable for the requirements of this project. This was because it was non-specific (reacting to a greater or lesser extent with all neem limonoids) and not stable over time (producing solutions in which absorption maximums were relatively transient). Consequently, chromatographic analysis would still be required to achieve a quantitative estimate of azadirachtin.

There is no further work that can be recommended with regard to improving the compatibility of this assay.

### **5.3. Chapter 3 overview – The development of a controlled-release granule**

Commercially available insecticides that contain azadirachtin are typically formulated as foliar applied, liquid sprays. However, on plant foliage, azadirachtin exhibits poor

insecticidal contact activity and a rapid degradation by photolysis. Therefore, opportunities exist for the introduction of alternative formulations, such as granules, which may improve the environmental stability and biological efficacy of applications. Granules can be defined as large, non-adhesive, dry particles that have become increasingly common due to the handling advantages they provide when compared to liquid sprays (Banks *et al.*, 1990; Hartley & Graham-Bryce, 1980b and Matthews, 2000). In addition, through controlled-release behaviour, this formulation type has often been observed optimising activity profiles while simultaneously limiting environmental contact (Barlow, 1985 and Hartley & Graham-Bryce, 1980b).

Based on these advantages, the aim of Chapter 3 was to investigate potential matrices that could be used to produce a granular formulation that exhibits controlled-release characteristics. This objective was achieved and several granular formulations were developed. However, not all of these produced a controlled, slow-release of azadirachtin. Based on this requirement, a notable success was granule matrices produced using sodium alginate.

There are a number of areas that will require additional work during further formulation development. In particular, more realistic release conditions should be considered. The studies conducted within this chapter examined release under conditions that are unlikely to reflect those occurring within the environment. Therefore, the release profiles observed (see figure 3.5.8) are possibly more representative of optimum rates, although for granules made from poly( $\epsilon$ -caprolactone), release may have been reduced by the methodology used. Consequently, a more valuable assessment may have been obtained if the release into



soil over time were studied. A second area that would benefit from further work regards assessing the stability of formulations within soils. This was unsuccessfully attempted during this project. Nevertheless, this may be particularly appropriate as results presented within Chapter 4 indicate that azadirachtin is not persistent within the soil environment.

#### **5.4. Chapter 4 overview – The movement of azadirachtin within soil and plant systems**

##### **5.4.1. The behaviour of azadirachtin within soils**

Unlike liquid applications that distribute material directly to the pest or its immediate environment, pesticides within granules are indirectly delivered to the target with final distribution of material occurring through local transport systems (Barlow, 1985). Within soils, this transport process has considerable potential of being modified through adsorption processes. This occurs primarily to organic matter and clay mineral components and is important because chemicals associated with the solid phase are generally immobile (Ding *et al.*, 2002; Hartley & Graham-Bryce, 1980a; Hesterberg, 1998 and Meyer-Windel & Lennartz, 1995).

Therefore, the first objective of Chapter 4 was to assess the likely mobility of azadirachtin within the soil. This research demonstrated that azadirachtin would be transported through the soil, dissolved within the soil water. During transport, the organic matter component of soils would be primarily responsible for adsorption of the limonoid. In contrast, clay minerals would be relatively non-sorbent. Nevertheless,

desorption from both of these locations would occur readily and overall, the limonoid can be classed as very highly soil mobile.

However, pesticides applied to the soil must exhibit not only appropriate mobility but also a level of molecular stability. Therefore, this chapter also studied this requirement. The results revealed that within the soil, azadirachtin's persistence would be relatively brief. When the pH of the soil water is mildly acidic or neutral, microbial activity is likely to be responsible for the limonoid's soil dissipation, with half-lives as short as 1.06 days and breakdown resulting in the acetyl moiety being cleaved from the molecule. However, within strongly alkaline soils, hydrolytic degradation will be mainly responsible for the breakdown of the limonoid, where the half-life of azadirachtin in solution may be as short as 7.15 hours at pH 9.

There are a few issues of this research that require further work. Primarily, the biological activity and environmental mobility/stability of the soil metabolite 3-deacetylazadirachtin should be determined. In addition, the breakdown route of azadirachtin within water requires identification.

#### 5.4.2. The behaviour of azadirachtin within plants

Pesticides that are transported dissolved within soil water are available for acquisition and passive absorption by roots. Following this process, long distance transport within a plant's vascular system can occur and this process itself takes place within the xylem and/or phloem vessels. The extent to which a xenobiotic moves within each of

these vessels can have important consequences for plant protection (Inoue *et al.*, 1998 and Kleier, 1994).

Therefore, the second objective of Chapter 4 was to investigate the systemic behaviour of azadirachtin within nasturtium plants using [ $^3\text{H}$ ]-dihydroazadirachtin as a reference. This study revealed that azadirachtin is both phloem and xylem mobile. Consequently, within nasturtium plants, the limonoid can be classified as ambimobile and will therefore be consumed by both general defoliators and sucking pests. In addition, the results of this chapter would predict that the level of plant protection offered by the limonoid should be relatively uniform throughout the leaf tissue of this plant. This can be proposed as azadirachtin was shown to accumulate within the leaf lamina in a nearly linear distribution. As with soils, not only is mobility important, but so too is compound stability. Following root absorption, it was observed that azadirachtin become very stable within nasturtium tissues, with a half-life of approximately 9 weeks. Within the plant, the route of metabolism is possibly similar to that which occurs in soils (i.e. cleavage of the acetyl moiety) or alternatively through conjugation with natural plant constituents.

There are a few areas regarding azadirachtin's behaviour within plants, which would benefit from further work. In particular, conclusively identifying the route of metabolism is an issue that needs to be addressed. Additionally, it is also important that the mobility and distribution of azadirachtin within other plant types (e.g. monocotyledons) and other tissues (e.g. fruits and flowers) are investigated.

#### 5.4.3. The movement of azadirachtin from granule to leaf tissues

The third and final objective of Chapter 4 was to follow the movement of azadirachtin, using [ $^3\text{H}$ ]-dihydroazadirachtin, from a granule source through the soil and into the leaf tissue. This work supported the predictions of the physico-chemical studies and directly confirmed azadirachtin's soil and plant mobility. In addition, this research also provided some indication as to the optimum granule placement strategy. This study suggested that granule position within soil will not effect the speed at which azadirachtin is released from the formulation. However, granule application method will affect the rate at which the limonoid is absorbed by roots and accumulated within the plant.

Possible areas for further work relate to variations regarding the soil and plant types used. Also, it would have been useful to determine the minimum soil moisture content that would permit effective granule release and root uptake. In addition, it would have been interesting if the insecticidal performance of the granular formulations had been investigated. This project did intend to study this effect and further the work initiated by Nisbet (1992), who used hydroponic conditions to load the plant *Nicotiana clevelandii* with azadirachtin, with subsequent antifeedant effects against the aphid *Myzus persicae*. However, due to time restrictions this was not possible.

#### 5.5. Concluding remarks

Despite extensive research that has investigated the insecticidal abilities of azadirachtin, the optimum method for delivering the limonoid to the target insect pest



remains undefined. In considering the physico-chemical behaviour of azadirachtin, it is somewhat puzzling to discover that all commercial formulations are applied to the foliage in liquid sprays and most often as emulsifiable concentrates. Clearly, the relationship between azadirachtin's aqueous and lipid solubility suggests that the penetration of the leaf cuticle will be limited, and let us not forget that the leaf structure is not designed to absorb solutes from the liquid phase. Additionally, the relationship between azadirachtin's aqueous and lipid solubility also suggests that the molecule will be relatively unsuccessful in crossing the insect cuticle to enable contact activity. As a consequence of this, for azadirachtin to produce a strong toxic effect in the target insect, the residue itself must be consumed. However, on the leaf surface, the rapid photolytic breakdown of azadirachtin will result in residues having an extremely limited persistence (Caboni *et al.*, 2002 and Schmutterer, 1990).

An obvious approach to address some of the problems mentioned above would be to apply azadirachtin to the soil in a granular formulation, as investigated in the research presented within this thesis. This type of formulation may confer a number of advantages including improved environmental persistence, as the limonoid is effectively shielded from the sun, and improved penetration into the crop, as uptake will be via the root. Consequently, a granular formulation may represent a more appropriate strategy for the application of azadirachtin against foliar plant pests such as aphids and lepidopterous larvae.

There are however, a number of alternative situations where this type of dry formulation may find a use. For example, neem-based granules may be applied to standing or stagnant water bodies in mosquito control programmes (US EPA, 2002).

The eggs and larvae of many species of mosquito develop at the water surface and therefore, granules that float may be used to specifically target these stages (Monmouth, 2003). Possible difficulties with this proposal relate to the speed of hydrolysis of the limonoid under alkaline conditions. However, in waters that are neutral or even mildly acidic, azadirachtin will exhibit a considerable persistence. In addition, it is also important to consider that one of azadirachtin's primary modes of action is through insect growth regulation (Mulla & Su, 1999). Therefore, it would seem that neem-based granules might be ideal candidates for disrupting the life cycle of the mosquito, so long as a reasonable level of photolytic stability can be achieved.

Granular formulations containing a neem extract or purified azadirachtin may also be useful for controlling cockroaches in homes, restaurants, hospitals, warehouses, offices and virtually any structure that has food preparation or storage areas. Granules may provide a convenient means of not only delivering a pesticide into hard-to-reach areas and hiding spaces, but also may be a useful practice that can limit the cockroach's ability to establish or reinvade. A further benefit of granules used under this application scenario is that there is considerable potential to change the matrix carrier, if for example, the palatability of the formulation needs to be improved, or alternatively, if the cockroaches learn to avoid the formulation.

Another potential use for neem-based granules could be in the control of various ant species. Granular formulations of the insecticides fipronil and deltamethrin are already used in such programs against species including the Argentine, Harvester, Leafcutter and Fire ant (AgrEvo, 1999 and Barr, 2003). In such situations, granules may be applied as perimeter treatments, to help prevent the infestation of buildings, or

alternatively, as a spot treatment to mounds where the objective is to kill the queen. During spot treatments of mounds, the fact that azadirachtin is not strongly bound to soil would suggest that the limonoid should successfully penetrate the nest following release from the granule.

Considering the potential importance that a number of authors are placing on azadirachtin, it is somewhat surprising to see such a limited amount of formulation types being proposed for the limonoid. While a majority of research has investigated simple foliar sprays and granules, there are a number of alternatives that may prove worthwhile for consideration.

Future work may look at the potential of using azadirachtin-containing microcapsules. Microcapsules of 3 $\mu$ m to 10 $\mu$ m can be effectively sprayed onto plant surfaces and may improve the persistence of deposits (Banks *et al.*, 1990 and Matthews, 2000). This form of preparation may be particularly suitable for azadirachtin, given that the limonoid exhibits a short persistence on leaf surfaces. However, it should also be considered that by formulating azadirachtin within microcapsules, the cost of any commercial formulation might be increased in comparison with more simple liquid sprays. However, it is possible that these costs may be offset by improved insect control.

Soil drenches that contain the limonoid may also be a useful means of distributing azadirachtin either directly to the pest, as in the case of soil dwelling insects, or alternatively to quickly supply a large amount of material for root absorption. This latter point may be important if an application is required in direct response to pest

presence. However, the slow acting nature of azadirachtin may realistically mean that this strategy is not acceptable. In addition, because azadirachtin is so highly soil mobile, it may be realistic to expect that the limonoid will be rapidly leached away from the areas where it is required, e.g. the rhizosphere.

Given the limited environmental persistence of azadirachtin, a particularly appropriate method of delivering the limonoid to the plant for systemic protection may be through a direct injection to stems or roots. This strategy is currently employed for delivering some insecticides, fungicides, antibiotics, plant growth regulators and mineral nutrients to trees (Tattar, 2003). However, an obvious drawback of this application strategy is that its suitability would be extremely limited, to for example, forestry situations.

The application of azadirachtin as a seed treatment is another strategy that is worthy of consideration. Numerous pesticides are applied to seed in order to provide protection during the vulnerable early stages of plant growth (Waller, 1985). However, for azadirachtin there may be problems regarding phytotoxicity when large amounts of the limonoid come into contact with the embryo. Additionally, there may be problems due to a restricted duration of effectiveness.

Clearly, there are a number of potential application strategies and formulations that can be proposed for various neem extracts and also for purified azadirachtin. It would also seem that a number of pests can be targeted in a variety of situations using neem derivatives. However, until there is a commercial need for these products, or a greater



desire for a botanically derived insecticide, both of which would subsequently drive research, these uses will remain very much, theoretical suggestions.

## **5.6. Conclusion**

The research presented within this thesis suggests that azadirachtin can easily be incorporated into various forms of granules. The release of the limonoid from these formulations has considerable capacity of being modified by using simple additives. The application of this type of formulation will require that local transport systems move the active substance from its source (i.e. granule) to its target (i.e. insects feeding on plant tissues). In this respect, azadirachtin was shown to be sufficiently mobile to achieve this. However, a major obstacle suggested by this project, is that the limonoid is rapidly degraded within the soil. Therefore, an insecticidal action may rely on the toxicity of the metabolite (3-deacetylazadirachtin). However, this metabolite may be less toxic than the parent and therefore, this will at best reduce the efficiency of controlling insect pests using neem formulations applied to the soil. In conclusion, based on the findings of this research, it is unclear if azadirachtin can make a commercially viable formulation for soil application due to an extremely rapid breakdown.

## References

**References**

Abou-Arab, A. A. K. (2002). Degradation of organochlorine pesticides by meat starter in liquid media and fermented sausage. Food Chem. Tox. 40: 33-41.

AgrEvo, (1999). Deltagard® G insecticide granule. AgrEvo Environmental Health, Inc. Product label.

Akou-Edi, D. (1983). Effects of neem seed powder and oil on *Tribolium confusum* and *Sitophilus zeamais*, pp445-452, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 2<sup>nd</sup> Int. Neem Conf. Rauschholzhausen, Germany.

Ambrosino, P., Fresa, R., Fogliano, V., Monti, S. M. & Ritieni, A. (1999). Extraction of azadirachtin A from neem seed kernels by supercritical fluid chromatography and its evaluation by HPLC and LC/MS. J. Agric. Food Chem. 47: 5252-5256.

Aminabhavi, T. M., Kulkarni, A. R. & Soppimath, K. S. (1999). Polymeric matrices for the release of bioactive agents. Polymer News. 24: 357-359.

Apra, C., Colosio, C., Mammone, T., Minoia, C. & Maroni, M. (2002). Biological monitoring of pesticide exposure: a review of analytical methods. J. Chrom. B. 769: 191-219.

Archer, D. W. & Shogren, J. F. (2001). Risk-indexed herbicide taxes to reduce ground and surface water pollution: an integrated ecological economics evaluation. Ecol. Econ. 38: 227-250.

Arnason, J. T., Philogène, B. J. R., Donskov, N., Hudon, M., McDougall, C., Fortier, G., Morand, P., Gardner, D., Lambert, J., Morris, C. & Nozzolillo, C. (1985). Antifeedant and insecticidal properties of azadirachtin to the European Corn Borer, *Ostrinia nubilalis*. Entomol. Exp. Appl. 38: 29-34.

Arpaia, S. & van Loon, J. J. A. (1993). Effects of azadirachtin after systemic uptake into *Brassica oleracea* on larvae of *Pieris brassicae*. Entomol. Exp. Appl. 66: 39-45.

- Ascher, K. R. S. (1981). Some physical (solubility) properties and biological (sterilant for *Epilachna varivestis* females) effects of a dried methanolic neem (*Azadirachta indica*) seed kernel extract, pp63-74, in Schmutterer, H., Ascher, K. R. S. & Rembold, H. (eds.) Proc. 1<sup>st</sup> Int. Neem Conf. Rottach-Egern.
- Ascher, K. R. S. (1993). Nonconventional insecticidal effects of pesticides available from the neem tree, *Azadirachta indica*. Arch. Insect. Biochem. Physiol. 22: 433-449.
- Ascher, K. R. S. (1997). Neem, eco-friendly, IPM-compatible plant derived bioinsecticide: some new findings. Dev. Plant Soil Sci. 71: 75-79.
- Avery, D. T. (1997). Saving the planet with pesticides, biotechnology and European farm reform, pp3-18 in Proceedings of the Brighton Crop Protection Conference – Weeds. The British Crop Protection Council, Farnham.
- Awasthy, K. S., Chaurasia, O. P. & Sinha, S. P. (1995). Genotoxic effects of crude extract of neem in bone marrow cells of mice. Cytologia. 60: 189-193.
- Ayas, Z., Barlas, N. E. & Kolankaya, D. (1997). Determination of organochlorine pesticide residues in various environments and organisms in Göksu Delta Turkey. Aquatic Tox. 39: 171-181.
- Banerjee, B. D. (1999). The influence of various factors on immune toxicity assessment of pesticide chemicals. Tox. Lett. 107: 21-31.
- Banks, A., Broadley, R., Collinge, M. & Middleton, K. (1990). Pesticide Application Manual (2nd Ed.). Queensland Department of Primary Industries, Brisbane.
- Barak, E., Dinoor, A. & Jacoby, B. (1983). Adsorption of systemic fungicides and a herbicide by some components of plant tissues, in relation to some physiochemical properties of the pesticides. Pestic. Sci. 14: 213-219.
- Barlow, F. (1985). Chemistry and formulation, pp1-34, in Haskell, P. T. (ed.) Pesticide Application: Principles and Practice. Clarendon Press, Oxford.



- Barnett, E. A., Fletcher, M. R., Hunter, K. & Sharp, E. A. (2002). Pesticide poisoning of animals 2001: investigations of suspected incidents in the United Kingdom. A Report of the Environmental Panel of the Advisory Committee on Pesticides. Central Science Laboratory, York., Scottish Agricultural Science Agency, Edinburgh.
- Barr, C. L. (2003). Speed of suppression of different carrier formulations of fipronil. Texas Imported Fire Ant Research and Management Project. <http://fireant.tamu.edu>
- Basedow, T. (2003). Effects of different neem preparations on homopterous pests and their stenophagous predators, pp1-7, in Cole, M. & Strang, R. (eds.) The Science & Application of Neem 2003.
- Beck, A. J. & Jones, K. C. (1996). The effects of particle size, organic matter content, crop residues and dissolved organic matter on the sorption kinetics of atrazine and isoproturon by clay soil. Chemosphere. 32: 2345-2358.
- Bei, J., He, W., Hu, X. & Wang, S. (2000). Photodegradation behaviour and mechanism of block copoly(caprolactone-ethylene glycol). Pol. Deg. Stab. 375-380.
- Benge, M. D. (1988). Cultivation and propagation of the neem tree, pp1-18 in Jacobson, M. (ed.) Focus on Phytochemical Pesticides (Vol. 1) The Neem Tree. CRC Press, Boca Raton, Florida.
- Berglöf, T., Dung, T. V., Kylin, H. & Nilsson, I. (2002). Carbendazim sorption-desorption in Vietnamese soils. Chemosphere. 48: 267-273.
- Bernard, C., Daberkow, S., Padgitt, M., Smith, M. E. & Uri, N. D. (1997). Alternative measures of pesticide use. Sci. Total Environ. 203: 229-244.
- Best, G. & Ruthren, D. (1995). Pesticides – developments, impacts, and controls. The Royal Society of Chemistry, Cambridge, UK.

- Betz, F. S., Hammond, B. G. & Fuchs, R. L. (2000). Safety and advantages of *Bacillus thuringiensis*-protected plants to control insect pests. Reg. Tox. Pharmacol. 32: 156-173.
- Bidmon, H. L., Kauser, G., Mobus, P. & Koolman, J. (1986). Effect of azadirachtin on blowfly larvae and pupae, pp253-271, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Bilton, J. N., Broughton, H. B., Jones, P. S., Ley, S. V., Lidert, Z., Morgan, E. D., Rzepa, H. S., Sheppard, R. N., Slawin, A. M. Z. & Williams, D. J. (1987). An x-ray crystallographic, mass spectroscopic, and nmr study of the limonoid insect antifeedant azadirachtin and related derivatives. Tetrahedron. 43: 2805-2815.
- Blaney, W. M. & Simmonds, M. S. J. (1990). A behavioural and electrophysiological study of the role of tarsal chemoreceptors in feeding by adults of Spodoptera, *Heliothis virescens* and *Helicoverpa armigera*. J. Ins. Physiol. 36: 743-756.
- Blaney, W. M. & Simmonds, M. S. J. (2002). Feeding behaviour, pp223-229, in Schmutterer, H. (ed.) The Neem Tree, *Azadirachta indica* A. Juss. and Other Meliaceous Plants. Sources of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes (2<sup>nd</sup> Ed.). Neem Foundation, Vithalnagar, Juhu Scheme, Mumbai, 2002.
- Blaney, W. M., Simmonds, M. S. J., Ley, S. V., Anderson, J. C. & Toogood, P. L. (1990). Antifeedant effects of azadirachtin and structurally related compounds on lepidopterous larvae. Entomol. Exp. Appl. 55: 149-160.
- Bokel, M., Cramer, R., Gutziet, H., Reeb, S. & Kraus, W. (1990). Tetranortriterpenoids related to nimbin and nimbolide from *Azadirachta indica* A. Juss (Meliacea). Tetrahedron. 46: 775-782.
- Bolognesi, C. & Morasso, G. (2000). Genotoxicity of pesticides: potential risk for consumers. Trends Food Sci. Tech. 11: 182-187.

- Boydston, R. A. (1992). Controlled release starch granule formulations reduce herbicide leaching in soil columns. Weed Tech. 6: 317-321.
- Brattsten, L. B. (1989). Insecticide resistance: research and management. Pestic. Sci. 26: 329-332.
- Bromilow, R. H. & Chamberlain, K. (1995). Principles governing uptake and transport of chemicals, pp37-38, in Trapp, S. & McFarlane, J. C. (eds.) Plant contamination: modelling and simulation of organic chemical processes. CRC Press, Boca Raton, Florida.
- Bromilow, R. H., Chamberlain, K. & Evans, A. A. (1990a). Physiochemical aspects of phloem translocation of herbicides. Weed Sci. 38: 305-314.
- Bromilow, R. H., Chamberlain, K. & Patil, S. G. (1990b). A rapid method using *Ricinus communis* for the estimation of phloem translocation of xenobiotics. Pestic. Sci. 30: 1-12.
- Bromilow, R. H., Evans, A. A. & Nicholls, P. H. (1999). Factors affecting degradation rates of five triazole fungicides in two soil types. I. Laboratory incubations. Pestic. Sci. 55: 1129-1134.
- Bromilow, R. H., Rigitano, R. L. O., Briggs, G. G. & Chamberlain, K. (1987). Phloem translocation of non-ionised chemicals in *Ricinus communis*. Pestic. Sci. 19: 85-99.
- Broughton, H. B., Ley, S. V., Slawin, A. M. Z., Williams, D. J. & Morgan, E. D. (1986). X-ray crystallographic structure determination of detigloyldihydroazadirachtin and reassignment of the structure of the limonoid insect antifeedant azadirachtin. J. Chem. Soc. Chem. Commun. 46-47.
- Buhler, D. D., Schreiber, M. M. & Koskinen, W. C. (1994a). Weed control with starch-encapsulated alachlor, metachlor, and atrazine. Weed Tech. 8: 277-284.

- Buhler, D. D., Koskinen, W. C., Schreiber, M. M. & Gan, J. (1994b). Dissipation of alachlor, metolachlor, and atrazine from starch-encapsulated formulations in a sandy loam soil. Weed Sci. 42: 411-417.
- Buterworth, J. H. & Morgan, E. D. (1968). Isolation of a substance that suppresses feeding in locusts. J. Chem. Soc. Chem. Commun. 23-24.
- Buterworth, J. H., Morgan, E. D. & Percy, G. R. (1972). The structure of azadirachtin: the functional groups. J. Chem. Soc. Perkin. Trans. 1: 2445-2446.
- Byrne, F. J. & Toscano, N. C. (2002). Evaluation of peracid activated organophosphates in studies of insecticide resistance conferred by insensitive acetylcholinesterases. J. Econ. Entomol. 95: 425-429.
- Caboni, P., Cabras, M., Angioni, A., Russo, M. & Cabras, P. (2002). Persistence of azadirachtin residues on olives after field treatment. J. Agric. Food Chem. 50: 3491-3494.
- California Environmental Protection Agency, Department of Pesticide Regulation. (2003). <http://www.cdpr.ca.gov>
- Campbell, N. A. (1993). Biology (3<sup>rd</sup> Ed.). The Benjamin/Cummings Publishing Company, Inc. California.
- Casanova, H., Ortiz, C., Peláez, C., Vallejo, A., Moreno, M. E. & Acevedo, M. (2002). Insecticide formulations based on nicotine oleate stabilized by sodium caseinate. J. Agric. Food Chem. 50: 6389-6394.
- Cayley, G. R. & Hide, G. A. (1980). Uptake of iprodione and control of diseases on potato stems. Pestic. Sci. 11: 15-19.
- Celis, R. & Koskinen, W. C. (1999). Characterization of pesticide desorption from soil by the isotopic exchange technique. Soil Sci. Soc. Am. J. 63: 1659-1666.



- Chafik, N., Dahchour, A. & Elamrani, M. K. (1998). Controlled release formulations of pesticides based on starch. Fresenius Envir. Bull. 7: 716-722.
- Chamberlain, K., Burell, M. M., Butcher, D. N. & White, J. C. (1984). Phloem transport of xenobiotics in *Ricinus communis* var. Gibsonii. Pestic. Sci. 15: 1-8.
- Champagne, D. E., Koul, O., Isman, M. B., Scudder, G. G. E. & Towers, G. H. N. (1992). Biological activity of limonoids from the rutales. Phytochemistry. 31: 377-394.
- Cheah, U. B., Kirkwood, R. C. & Lum, K. Y. (1997). Adsorption, desorption and mobility of four commonly used pesticides in Malaysian agricultural soils. Pestic. Sci. 50: 53-63.
- Chen, S., Petersen, B. L., Olsen, C. E., Schulz, A. & Halkier, B. A. (2001). Long-distance phloem transport of glucosinolates in arabidopsis. Plant Physiol. 127: 194-201.
- Chopra, R. (1928). Annual report of the entomologist to the government, Punjab, 1925-26. Rep. Dep. Agric. Punjab. 1: 67-125.
- Choudary, B. M., Prasad, B. P. & Kantam, M. L. (1989). New Interlamellar pesticide-metal-montmorillonite complexes: a novel technique for controlled release. J. Agric. Food Chem. 37: 1422-1425.
- Chowdhury, A. N. U., Jepson, P. C., Howse, P. E. & Ford, M. G. (2001). Leaf surfaces and the bioavailability of pesticide residues. Pest Manag. Sci. 57: 403-412.
- Clarke, J. H., Clark, W. S. & Hancock, M. (1997). Strategies for the prevention of development of pesticide resistance in the UK – lessons for and from the use of herbicides, fungicides and insecticides. Pestic. Sci. 51: 391-397.

- Coats, J. R. (1990). Mechanisms of toxic action and structure-activity relationships for organochlorine and synthetic pyrethroid insecticides. Environ. Health Perspect. 87: 255-62.
- Coats, J. R. (1994). Risks from natural versus synthetic insecticides. Annu. Rev. Entomol. 39: 489-515.
- Cochran, R. C. (2002). Appraisal of risks from nonoccupational exposure to chlorpyrifos. Reg. Tox. Pharmacol. 35: 105-121.
- Cole, D. J. (1994). Detoxification and activation of agrochemicals in plants. Pestic. Sci. 42: 209-222.
- Collins, R. L. & Doglia, S. (1973). Concentration of pesticides slowly released by diffusion. Weed Sci. 21: 343-349.
- Collins, R. L., Doglia, S., Mazak, R. A. & Samulski, E. T. (1973). Controlled release of herbicides – Theory. Weed Sci. 21: 1-5.
- Colosio, C., Corsini, E., Barcellini, W. & Maroni, M. (1999). Immune parameters in biological monitoring of pesticide exposure: current knowledge and perspectives. Tox. Lett. 108: 285-295.
- Colosio, C., Tiramani, M. & Maroni, M. (2003). Neurobehavioral effects of pesticides: state of the art. NeuroToxicology. In Press.
- Commision Directive 92/69/EEC (1992). Annex to Commision Directive 92/69/EEC of 31 July 1992 adapting to technical progress for the seventeenth time Council Directive 67/548/EEC on the approximation of laws, regulations and administrative provisions relating to the classification, packaging and labelling of dangerous substances. Official J. Europe. Commun. 35: 29 December 1992.
- Commision of the European Communities (1997). Metabolism and distribution in plants. Directorate General for Agriculture. 22/7/97. Review 3: 7028/VI/95.

Connick, W. J. (1982). Controlled release of the herbicides 2,4-D and dichlobenil from alginate gels. J. Appl. Pol. Sci. 27:3341-3348.

Connick, W. J. (1988). Formulation of living biological control agents with alginate, pp241-250, in Cross, B. & Scher, H. B. (eds.) Pesticide Formulations: Inovations and Developments. American Chemical Society.

Connick, W. J., Bradow, J. M., Wells, W., Steward, K. K. & Van, T. K. (1982). Release of dichlobenil from clay-filled alginate and carboxymethylcellulose gel granules. Book of abstracts, 9th International Symposium on Controlled Release of Bioactive Materials. Ft. Lauderdale, Florida. July 25-28, pp46-48.

Connick, W. J., Bradow, J. M., Wells, W., Steward, K. K. & Van, T. K. (1984). Preparation and evaluation of controlled-release formulations of 2,6-dichlorobenzonitrile. J. Agric. Food Chem. 32: 1199-1205.

Copping, L. G. & Menn, J. J. (2000). Biopesticides: a review of their action, applications and efficacy. Pest Manag. Sci. 56: 651-676.

Cotterill, J. V., Wilkins, R. M. & da Silva, F. T. (1996). Controlled release of diuron from granules based on a lignin matrix system. J. Controlled Release. 40: 133-142.

Coudriet, D. L., Prabhaker, N. & Meyerdirk, D. E. (1985). Sweet potato whitefly (Homoptera: Aleyrodidae): effect of neem seed extract on oviposition and immature stages. Environ. Entomol. 14: 776-779.

Council Directive 91/414/EEC (1991). Council directive of 15 july 1991 concerning the placing of plant protection products on the market. Official J. Europe. Commun. L 230: 1-32.

Cox, L., Walker, A. & Welch, S. J. (1996). Evidence for the accelerated degradation of isoproturon in soils. Pestic. Sci. 48: 253-260.

- Crafts, A. S. & Yamaguchi, S. (1960). Absorption of herbicides by roots. Amer. J. Bot. 47: 248-255.
- Cryer, S. A. & Laskowski, D. A. (1998). Chlorpyrifos release rate from clay granules: experimental observations and simple algorithm development for use in computer-based exposure assessments. J. Agric. Food. Chem. 46: 3810-3816.
- Dai, S., Liu, G., Qian, Y. & Cheng, X. (2001a). The sorption behaviour of complex pollution system composed of aldicarb and surfactant-SDBS. Wat. Res. 35: 2286-2290.
- Dai, J., Yaylayan, V. A., Raghavan, G. S. V. & Parè, J. C. (1999). Extraction and colorimetric determination of azadirachtin-related limonoids in neem seed kernels. J. Agric. Food Chem. 47: 3738-3742.
- Dai, J., Yaylayan, V. A., Raghavan, G. S. V., Parè, J. C. & Liu, Z. (2001b). Multivariate calibration for the determination of total azadirachtin-related limonoids and simple terpenoids in neem extracts using vanillin assay. J. Agric. Food Chem. 49: 1169-1174.
- Darwis, D., Mitomo, H., Enjoji, T., Yoshii, F. & Makuuchi, K. (1998). Enzymatic degradation of radiation crosslinked poly( $\epsilon$ -caprolactone). Pol. Deg. Stab. 62: 259-265.
- Das, A. C. & Mukherjee, D. (2000). Soil application of insecticides influences microorganisms and plant nutrients. Appl. Soil Ecol. 14: 55-62.
- Davis, R. F., Wauchope, R. D., Johnson, A. W., Burgoa, B. & Pepperman, A. B. (1996). Release of fenamiphos, atrazine, and alachlor into flowing water from granules and spray deposits of conventional and controlled-release formulations J. Agric. Food Chem. 44: 2900-2907.



- Dejonckheere, W., Steurbaut, W., Melkebeke, G. & Kips, R. H. (1983). Leaching of aldicarb and thiofanox, and their uptake in soils by sugarbeet plants. Pestic. Sci. 14: 99-107.
- Denyer, R. (2000). Introduction: integrated crop management (ICM). Pest Manag. Sci. 56: 945-946.
- Dethier, V. G. (1956). Repellents. Annu. Rev. Entomol. 1: 181-202.
- Dev, S. & Koul, O. (1997). Insecticides of natural origin. Harwood academic publishers, Netherlands.
- Devine, M. D. & Hall, L. M. (1990). Implications of sucrose transport mechanisms for the translocation of herbicides. Weed Sci. 38: 299-304.
- Ding, G., Novak, J. M., Herbert, S. & Xing, B. (2002). Long-term tillage effects on soil metolachlor sorption and desorption behaviour. Chemosphere. 48: 897-904.
- Dorn, A., Rademacher, J. M. & Sehn, E. (1986). Effects of azadirachtin on reproductive organs and fertility in the large milkweed bug, *Oncopeltus fasciatus*, pp273-288, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Durand-Réville, T., Ley, S. V., Gobbi, L., Scott, J. S. & Somers, A. Q. (2001). Towards the total synthesis of azadirachtin, pp10-13, in Cole, M. & Strang, R. (eds.) The Science and Application of Neem 2001. Glasgow.
- ECPA (1998a). Registering crop protection products in the EU: a technical view. European Crop Protection Association. Brussels, 1998.
- ECPA (1998b). Approval of crop protection products within the EU. European Crop Protection Association. Brussels, 1998.

ECPA (2000a). Crop protection products in and around European towns and cities. Protecting our amenities from pests and diseases. European Crop Protection Association. Brussels, 2000.

ECPA (2000b). ECPA proposal for the establishment of a central evaluation unit for PPPs active substances within the proposed European food authority (EFA). European Crop Protection Association (position paper). Brussels, 6 October 2000.

ECPA (2001a). ECPA view on the July 2001 commission report to the European Parliament and the council on the EU review programme under directive 91/414/EEC. European Crop Protection Association (position paper). Brussels, 22 March 2001.

ECPA (2001b). Impact of current legislation on industry competitiveness and innovation. European Crop Protection Association (position paper). Brussels, 15 June 2001.

Edelson, J. V., Duthie, J. & Roberts, W. (2002). Toxicity of biorational insecticides: activity against the green peach aphid, *Myzus persicae* (Sulzer). Pest Mang. Sci. 58: 255-260.

Edgington, L. V. (1981). Structural requirements of systemic fungicides. Ann. Rev. Phytopathol. 19: 107-124.

Edwards, V. T. McMinn, A. L. & Wright, A. N. (1982). Sugar conjugates of pesticides and their metabolites in plants – current status, pp71-125, in Hutson, D. A. & Roberts, T. R. (eds.) Progress in Pesticide Biochemistry (Vol. 2). John Wiley & Sons, Chichester, New York, Brisbane, Toronto, Singapore.

Edwards-Jones, G. & Howells, O. (2001). The origin and hazard of inputs to crop protection in organic farming systems: are they sustainable? Agric. Syst. 67: 31-47.

Ellgehausen, H., D'Hondt, C. & Fuerer, R. (1981). Reversed-phase chromatography as a general method for determining octan-1-ol/water partition coefficient. Pestic. Sci. 12: 219-227.

- Enriz, R. D., Baldoni, H. A., Zamora, M. A., Jáuregui, E. A., Sosa, M. E., Tonn, C. E., Luco, J. M. & Gordaliza, M. (2000). Structure – antifeedant activity relationship of clerodane diterpenoids. Comparative study with anolides and azadirachtin. J. Agric. Food Chem. 48: 1384-1392.
- Ermel, K., Pahlich, E. & Schmutterer, H. (1983). Comparison of the azadirachtin content of neem seeds from ecotypes of Asian and African origin, pp91-94, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 2<sup>nd</sup> Int. Neem Conf. Rauischholzhausen, Germany.
- Europa (2002). 320 pesticides to be withdrawn in July 2003. EU Institution Press Release. 4 July 2002.
- Europa (2003). Commission close to completion of pesticide review: 110 additional substances to be withdrawn. EU Institution Press Release. 8 July 2003.
- European Commission (2000). Guidance document on persistence in soil. Directorate General for Agriculture. 12 July 2000.
- Fagoonee, I. (1986). Use of neem in vegetable crop protection in Mauritius, pp419-429, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Fakata, K. L., Swanson, S. A. Vorce, R. L. & Stemmer, P. M. (1998). Pyrethroid insecticides as phosphatase inhibitors. Biochem. Pharmacol. 55: 2017-2022.
- Felding, G. (1997). Pesticide adsorption as a function of depth below surface. Pestic. Sci. 50: 64-66.
- Feng, R. & Isman, M. B. (1995). Selection for resistance to azadirachtin in the green peach aphid, *Myzus persicae*. Experientia. 51: 831-833.
- Fisher, D. B. (1978). An evaluation of the Münch hypothesis for phloem transport in soybean. Planta. 139: 25-28.

- Fleming, G. W., Simmons, F. W., Wax, L. M., Wing, R. E. & Carr, M. E. (1992). Atrazine movement in soil columns as influenced by starch-encapsulation and acrylic polymer additives. Weed Sci. 40: 465-470.
- Furmidge, C. G. L. (1984). Formulation and application factors involved in the performance of soil-applied pesticides, pp49-64, in Hance, R. J. (ed.) Soils and Crop Protection Chemicals. Monograph No. 27. Proceedings of a symposium held at Wye College, Kent. 11<sup>th</sup> and 12<sup>th</sup> July, 1984. BCPC Publications, Croydon.
- Galloway, T. S., Millward, N., Browne, M. A. & Depledge, M. H. (2002). Rapid assessment of organophosphorus/carbamate exposure in the bivalve mollusc *Mytilus edulis* using combined esterase activities as biomarkers. Aquatic Tox. 61: 169-180.
- Gan, J., Hussain, M. & Rathor, N. M. (1994). Behaviour of an alginate-kaolin based controlled-release formulation of the herbicide thiobencarb in simulated ecosystems. Pestic. Sci. 42: 265-272.
- Garcia, S. S., Ake, C., Clement, B., Huebner, H. J., Donnelly, K. C. & Shalat, S. L. (2001). Initial results of environmental monitoring in the Texas Rio Grande valley. Environ. Int. 26: 465-474.
- Garden Gate (2003). Soil Basics: pH and Nutrients. <http://www.gardengatemagazine.com>
- Gardner, L. R. (1953). Pesticide formulations: basic principles of formulations. Agric. Food Chem. 1: 521-522.
- Gawlik, B. M., Kettrup, A. & Muntau, H. (1999). Characterisation of a second generation of European reference soils for sorption studies in the framework of chemical testing – part II: soil adsorption behaviour of organic chemicals. Sci. Total Environ. 229: 109-120.



- Gawlik, B. M., Sotiriou, N., Feicht, E. A., Schulte-Hostede, S. & Kettrup, A. (1997). Alternatives for the determination of the soil adsorption coefficient, K<sub>oc</sub>, of non-ionic organic compounds – a review. Chemosphere. 34: 2525-2551.
- Gerstl, Z. (2000). An update of the K<sub>oc</sub> concept in regard to regional scale management. Crop Prot. 19: 643-648.
- Gómez-Arroyo, S., Díaz-Sánchez, Y., Meneses-Pérez, M. A., Villalobos-Pietrini, R. & León-Rodríguez, J. D. (2000). Cytogenetic biomonitoring in a Mexican floriculture worker group exposed to pesticides. Mutation Res. 466: 117-124.
- Gong, A., Ye, C., Wang, X., Lei, Z. & Liu, J. (2001). Dynamics and mechanism of ultraviolet photolysis of atrazine on soil surface. Pest Manag. Sci. 57: 380-385.
- Govindachari, T. R. (1992). Chemical and biological investigations on *Azadirachta indica* (the neem tree). Current Sci. 63: 117-122.
- Govindachari, T. R., Sandhya, G. & Raj, S. P. G. (1992a). Azadirachtins H and I: two new tetranortriterpenoids from *Azadirachta indica*. J. Nat. Prod. 55: 596-601.
- Govindachari, T. R., Sandhya, G. & Raj, S. P. G. (1992b). Structure of azadirachtin K, a new tetranortriterpenoid from *Azadirachta indica*. Ind. J. Chem. 31B: 295-298.
- Grace, J. K. & Yates, J. R. (1992). Behavioural effects of a neem insecticide on *Coptotermes formosanus* (Isoptera: Rhinotermitidae). Tropical Pest Manag. 38: 176-180.
- Gramatica, P., Corradi, M. & Consonni, V. (2000). Modelling and prediction of soil sorption coefficients of non-ionic organic pesticides by molecular descriptors. Chemosphere. 41: 763-777.
- Gramatica, P. & Guardo, A. D. (2002). Screening of pesticides for environmental partitioning tendency. Chemosphere. 47: 947-956.

- Grayson, B. T. & Kleier, D. A. (1990). Phloem mobility of xenobiotics. IV. Modelling of pesticide movement in plants. Pestic. Sci. 30: 67-79.
- Guo, L., Jury, W. A., Wagenet, R. J. & Flury, M. (2000). Dependence of pesticide degradation on sorption: nonequilibrium model and application to soil reactors. J. Contam. Hydrol. 43: 45-62.
- Hamers, T., van den Brink, P. J., Mos, L., van der Linden, S. C., Legler, J., Koeman, J. H. & Murk, A. J. (2003). Estrogenic and esterase – inhibiting potency in rainwater in relation to pesticide concentrations, sampling season and location. Environ. Pollution. 123: 47-65.
- Hartley, G. S. & Graham-Bryce, I. J. (1980a). The dynamics of applied pesticides in the local environment in relation to biological response (Vol. 1). Academic Press, London.
- Hartley, G. S. & Graham-Bryce, I. J. (1980b). Physical Principles of Pesticide Behaviour. The Dynamics of Applied Pesticides in the Local Environment in Relation to Biological Response (Vol. 2). Academic Press, London.
- Hassall, K. A. (1969). World crop protection (Vol. 2). Illife Books, London.
- Hassanali, A. & Bentley, M. D. (1986). Comparison of the insect antifeedant activities of some limonoids, pp683-689, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Heimbach, U., Kral, G. & Niemann, P. (2002). EU regulatory aspects of resistance risk assessment. Pest Manag. Sci. 58: 935-938.
- Hein, D. F. & Hummel, H. E. (2000). MLCCC: a promising technique for the isolation of pesticides of plant origin. <http://chemsoc.org/chempest/html/3A-0039.html>

Hellpap, C. & Dreyer, M. (1995). Neem products for pest management, practical results for neem application against arthropod pests, and probability of development of resistance, pp367-375, in Schmutterer, H. (ed.) The Neem Tree, *Azadirachta indica* A. Juss. and Other Meliaceae Plants. Sources of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes. VCH Verlagsgesellschaft mbH, Germany.

Henderson, R. A., McCrindle, R., Melera, A. & Overton, K. H. (1968). Tetranortriterpenoids – IX. The constituents and stereochemistry of salannin. Tetrahedron. 24: 1525-1528.

Hermosin, M. C., Calderón, M. J., Aguer, J. P. & Cornejo, J. (2001). Organoclays for controlled release of the herbicide fenuron. Pest Manag. Sci. 57: 803-809.

Hernández, F., Serrano, R., Roig-Navarro, A. F., Martínez-Bravo, Y. & López, F. J. (2000). Persistent organochlorines and organophosphorus compounds and heavy elements in common whale (*Balaenoptera physalus*) from the western Mediterranean sea. Marine Pollution Bulletin. 40: 426-433.

Hesterberg, D. (1998). Biogeochemical cycles and processes leading to changes in mobility of chemicals in soils. Agric. Eco. Environ. 67: 121-133.

Heudorf, U. & Angerer, J. (2001). Metabolites of organophosphorous insecticides in urine specimens from inhabitants of a residential area. Environ. Res. Section A. 86: 80-87.

Hill, D. S. (1994). Agricultural entomology. Timber Press, Oregon USA, Cambridge UK.

Hopkins, W. G. (1999). Introduction to Plant Physiology (2<sup>nd</sup> Ed.). John Wiley & Sons, New York, Chichester, Weinheim, Brisbane, Singapore, Toronto.

Hsu, F. C. Kleier, D. A. & Melander, W. R. (1988). Phloem mobility of xenobiotics. II. Bioassay testing of the unified mathematical model. Plant Physiol. 86: 811-816.

- Huang, X. & Lee, L. S. (2001). Organic compounds in the environment. Effects of dissolved organic matter from animal waste effluent on chlorpyrifos sorption by soils. J. Environ. Qual. 30: 1258-1265.
- Hunter, W. B. & Ullman, D. E. (1992). Effects of the neem product, RD-Repelin, on settling behaviour and transmission of zucchini yellow mosaic virus by the pea aphid, *Acyrtosiphon pisum* (Harris) (Homoptera: Aphididae). Ann. Appl. Biol. 120: 9-15.
- Hussain, M., Gan, J. & Rathor, M. N. (1992). Preparation of controlled-release formulations of  $^{14}\text{C}$ -labelled thiobencarb herbicide and study of their environmental behaviour. Pestic. Sci. 34: 341-347.
- Immaraju, J. A. (1998). The commercial use of azadirachtin and its integration into viable pest control programmes. Pestic. Sci. 54: 285-289.
- Inoue, J., Chamberlain, K. & Bromilow, R. H. (1998). Physiochemical factors affecting the uptake by roots and translocation to shoots of amine bases in barley. Pestic. Sci. 54: 8-21.
- Isman, M. B. (2000). Plant essential oils for pest and disease management. Crop Prot. 19: 603-608.
- Isman, M. B., Koul, O., Luczynski, A. & Kaminski, J. (1990). Insecticidal and antifeedant bioactivities of neem oils and their relationship to azadirachtin content. J. Agric. Food Chem. 38: 1406-1411.
- Jacobson, M. (1986). Neem research and cultivation in the western hemisphere, pp33-44, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Jacobson, M. (1988). Pharmacology and toxicology of neem, pp133-154, in Jacobson, M. (ed.) Focus on Phytochemical Pesticides (Vol. 1) The Neem Tree. CRC Press, Boca Raton, Florida.



- Jaglan, M. S., Khokhar, K. S., Malik, M. S. & Singh, R. (1997). Evaluation of neem (*Azadirachta indica* A. Juss) extracts against American bollworm, *Helicoverpa armigera* (Hubner). J. Agric. Food Chem. 45: 3262-3268.
- Jarvis, A. P., Johnson, S. & Morgan, E. D. (1998). Stability of the natural insecticide azadirachtin in aqueous and organic solvents. Pestic. Sci. 53: 217-222.
- Jarvis, A. P., Morgan, E. D. & Edwards, C. (1999). Rapid separation of triterpenoids from neem seed extracts. Phytochem. Anal. 10: 39-43.
- Johnson, A. C., Bettinson, R. J. & Williams, R. J. (1999). Differentiating between physical and chemical constraints on pesticide and water movement into and out of soil aggregates. Pestic. Sci. 55: 524-530.
- Johnson, R. M. & Pepperman, A. B. (1995). Soil column mobility of metribuzin from alginate-encapsulated controlled release formulations. J. Agric. Food Chem. 43: 241-246.
- Johnson, R. M. & Pepperman, A. B. (1996). Leaching of alachlor from alginate-encapsulated controlled-release formulations. Pestic. Sci. 48: 157-164.
- Johnson, R. M. & Pepperman, A. B. (1998). Release of atrazine and alachlor from clay-oxamide controlled-release formulations. Pestic. Sci. 53: 233-240.
- Jones, P. S., Ley, S. V., Morgan, E. D. & Santafianos, D. (1988). The chemistry of the neem tree, pp19-45, in Jacobson, M. (ed.) Focus on Phytochemical Pesticides (Vol. 1) The Neem Tree. CRC Press, Boca Raton, Florida.
- Jotwani, M. C. & Srivastava, K. P. (1983). A review of neem research in India in relation to insects, pp43-56, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 2<sup>nd</sup> Int. Neem Conf. Rauischholzhausen, Germany.
- Kakareka, S. V. (2002). Sources of persistent organic pollutants emission on the territory of Belarus. Atmos. Environ. 36: 1407-1419.

- Karel, A. K. (1986). Response of *Ootheca bennigseni* (Coleoptera : Chrysomelidae) to neem extracts, pp393-403, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Keifer. M. C. (2000). Effectiveness of interventions in reducing pesticide overexposure and poisonings. Am. J. Prev. Med. 18: 80-89.
- Kenawy, E. R. (1998). Recent advances in controlled release of agrochemicals. J. M. S. – Rev. Macromol. Chem. Phys. 38: 365-390.
- Kenawy, E. R. & Sakran, M. A. (1996). Controlled release formulations of agrochemicals from calcium alginate. Ind. Eng. Chem. Res. 35: 3726-3729.
- Ketar, C. M. (1986). Use of tree-derived non-edible oils as surface protectants for stored legumes against *Callosobruchus maculatus* and *C. chinensis*, pp525-542, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Khan, M. Q. (1987). Studies on the measurement of extractable and mineralizable nitrogen in soil. Ph. D. Thesis. University of Glasgow, Glasgow.
- King, R. W. & Zeevaart, J. A. D. (1974). Enhancement of phloem exudation from cut petioles by chelating agents. Plant Physiol. 53: 96-103.
- Kirkwood, R. C. (1987). Uptake and movement of herbicides from plant surfaces and the effects of formulation and environment upon them, pp1-25, in Cottrell, H. J. (ed.) Critical Reports on Applied Chemistry (Vol. 18). Pesticides on Plant Surfaces, John Wiley & Sons, Chichester, New York, Brisbane, Toronto, Singapore.
- Kirkwood, R. C. (1999). Recent developments in our understanding of the plant cuticle as a barrier to the foliar uptake of pesticides. Pestic. Sci. 55: 69-77.
- Kirsh, K. (1986). Studies of the efficacy of neem extracts in controlling major insect pests in tobacco and cabbage, pp495-515, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 2<sup>nd</sup> Int. Neem Conf. Rauschholzhausen, Germany.

- Kleier, D. A. (1988). Phloem mobility of xenobiotics. 1. Mathematical model unifying the weak acid and intermediate permeability theories. Plant Physiol. 86: 803-810.
- Kleier, D. A. (1994). Phloem mobility of xenobiotics. V. Structural requirements for phloem-systemic pesticides. Pestic. Sci. 42: 1-11.
- Kleivane, L., Severinsen, T. & Skaare, J. U. (2000). Biological transport and mammal to mammal transfer of organochlorines in Arctic fauna. Marine Environ. Res. 49: 343-357.
- Klemm, U. & Schmutterer, H. (1993). Effects of neem preparations on *Plutella xylostella* L. and its natural enemies of the genus *Trichogramma*. Z. Pflanzenkr. Pflanzenschutz. 100: 113-128.
- Kogan, M. & Bayer, D. E. (1996). Herbicide uptake as influenced by plant water status. Pestic. Biochem. Physiol. 56: 174-182.
- Konstantinou, I. K., Goutner, V. & Albanis, T. A. (2000). The incidence of polychlorinated biphenyl and organochlorine pesticide residues in the eggs of the cormorant (*Phalacrocorax carbo sinensis*): an evaluation of the situation in four Greek wetlands of international importance. Sci. Total Environ. 257: 61-79.
- Koul, O. (1984). Azadirachtin I. Interaction with the development of red cotton bugs. Entomol. Exp. Appl. 36: 85-88.
- Koul, O., Isman, M. B. & Ketar, C. M. (1990). Properties and uses of neem, *Azadirachta indica*. Can. J. Bot. 68: 1-11.
- Kousba, A. A., Poet, T. S. & Timchalk, C. (2003). Characterization of the in vitro kinetic interaction of chlorpyrifos-oxon with rat salivary cholinesterase: a potential biomonitoring matrix. Toxicology. 188: 219-232.

- Kraus, W. (2002). Biologically active ingredients, azadirachtin and other triterpenoids – Parts 1 & 2, pp39-98, in Schmutterer, H. (ed.) The Neem Tree, *Azadirachta indica* A. Juss. and Other Meliaceae Plants. Sources of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes (2<sup>nd</sup> Ed.). Neem Foundation, Vithalnagar, Juhu Scheme, Mumbai, 2002.
- Kraus, W., Bokel, M., Bruhn, A., Cramer, R., Klaiber, I., Klenk, A., Nagl, G., Pöhl, H., Sadlo, H. & Vogler, B. (1987). Structure determination by nmr of azadirachtin and related compounds from *Azadirachta indica* A. Juss (Meliaceae). Tetrahedron. 43: 2817-2830.
- Kraus, W., Bokel, M., Klenk, A. & Pöhl, H. (1985). The structure of azadirachtin and 22, 23-dihydro-23 $\beta$ -methoxyazadirachtin. Tetrahedron Lett. 26: 6435-6438.
- Kreutzweiser, D. P., Back, R. C., Sutton, T. M., Thompson, D. G. & Scarr, T. A. (2002). Community-level disruptions among zooplankton of pond mesocosms treated with a neem (azadirachtin) insecticide. Aquatic Tox. 56: 257-273.
- Krol, W. J., Arsenault, T. L., Pylypiw (Jr), H. M. & Mattina, M. J. I. (2000). Reduction of pesticide residues on produce by rinsing. Agric. Food Chem. 48: 4666-4670.
- Kulkarni, A. R., Soppimath, K. S. & Aminabhavi, T. M. (1999). Solubility study of *Azadirachta indica* A. Juss. (neem) seed oil in the presence of cosolvent/nonionic surfactant at (298.15, 303.15, 308.15, and 313.15) K. J. Chem. Eng. Data. 44: 836-838.
- Kulkarni, A. R., Soppimath, K. S., Aminabhavi, T. M., Dave, A. M. & Mehta, M. H. (2000). Glutaraldehyde crosslinked sodium alginate beads containing liquid pesticide for soil application. J. Controlled Release. 63: 97-105.
- Kumar, J. & Parmar, B. S. (1996). Physicochemical and chemical variation in neem oils and some bioactivity leads against *Spodoptera litura* F. J. Agric. Food Chem. 44: 2137-2143.



- Kumar, L. & Parmar, B. S. (2000). Effect of emulsion size and shelf life of azadirachtin A on the bioefficacy of neem (*Azadirachta indica* A. Juss) emulsifiable concentrates. J. Agric. Food Chem. 48: 3666-3672.
- Kunisue, T., Watanabe, M., Subramanian, A., Sethuraman, A., Titenko, A. M., Qui, V., Prudente, M. & Tanabe, S. (2003). Accumulation features of persistent organochlorines in resident and migratory birds from Asia. Environ. Pollution. 125: 157-172.
- Kydonieus, A. F. (1980). Fundamental concepts of controlled release, pp1-19, in Kydonieus, A. F. (ed.) Controlled Release Technologies, Methods, Theory and Applications (Volume 1). CRC Press, Boca Raton, Florida.
- Laden, F., Hankinson, S. E., Wolff, M. S., Colditz, G. A., Willett, W. C., Speizer, F. E. & Hunter, D. J. (2001). Plasma organochlorine levels and the risk of breast cancer: and extended follow-up in the nurses' health study. Int. J. Cancer. 91: 568-574.
- Leake, A. (2000). The development of integrated crop management in agricultural crops: comparisons with conventional methods. Pest Manag. Sci. 56: 950-953.
- Leake, C. R. & Gatzweiler, E. W. (1995). The relative importance of adsorption and desorption constants to the assessment of pesticide leaching potential in soil, pp19-24, in Walker, A., Allen, R., Bailey, S. W., Blair, A. M., Brown, C. D., Günther, P., Leake, C. R. & Nicholls, P. H. (eds.) Pesticide Movement to Water. BCPC Monograph No. 62. British Crop Protection Council, Surrey, UK.
- Leake, C. R., Humphreys, S. P. & Austin, D. J. (1995). Factors influencing the modelling of pesticide degradation in soil and the estimation of half-life (DT<sub>50</sub>) and DT<sub>90</sub> values, pp195-200, in Walker, A., Allen, R., Bailey, S. W., Blair, A. M., Brown, C. D., Günther, P., Leake, C. R. & Nicholls, P. H. (eds.) Pesticide Movement to Water. BCPC Monograph No. 62. British Crop Protection Council, Surrey, UK.
- Lee, P. I. (1985). Kinetics of drug release from hydrogel matrices. J. Controlled Release. 2: 277-288.

- Lee, S. E. & Lees, E. M. (2001). Biochemical mechanisms of resistance in strains of *Oryzaephilus surinamensis* (Coleoptera: Silvanidae) resistant to malathion and chlorpyrifos-methyl. J. Econ. Entomol. 94: 706-713.
- Leeds-Harrison, P. B. (1995). The movement of water and solutes to surface and ground waters, pp3-12, in Walker, A., Allen, R., Bailey, S. W., Blair, A. M., Brown, C. D., Günther, P., Leake, C. R. & Nicholls, P. H. (eds.) Pesticide Movement to Water. BCPC Monograph No. 62. British Crop Protection Council, Surrey, UK.
- Lesan, H. M. & Bhandari, A. (2003). Atrazine sorption on surface soils: time-dependent phase distribution and apparent desorption hysteresis. Water Res. 37: 1644-1654.
- Lewalter, J. & Leng, G. (1999). Consideration of individual susceptibility in adverse pesticide effects. Tox. Lett. 107: 131-144.
- Ley, S. V., Anderson, J. C., Blaney, W. M., Jones, P. S., Lidert, Z., Morgan, E. D., Robinson, N. G., Santafianos, D., Simmonds, M. S. J. & Toogood, P. L. (1989a). Insect antifeedants from *Azadirachta indica* (Part 5)<sup>1</sup>: Chemical modification and structure-activity relationships of azadirachtin and some related limonoids. Tetrahedron. 45: 5175-5192.
- Ley, S. V., Anderson, J. C., Blaney, W. M., Lidert, Z., Morgan, E. D., Robinson, N. G. & Simmonds, M. S. J. (1988). Chemistry of insect antifeedants from *Azadirachta indica* (Part 3)<sup>1</sup>: Reactions on the C-22, 23 enol ether double bond of azadirachtin and conversion to 22, 23-dihydro-23- $\beta$ -methoxyazadirachtin. Tetrahedron Lett. 29: 5433-5436.
- Ley, S. V., Anderson, J. C., Blaney, W. M., Morgan, E. D., Sheppard, R. N., Simmonds, M. S. J., Slawin, A. M. Z., Simth, S. C., Williams, D. J. & Wood, A. (1991). Chemistry of insect antifeedants from *Azadirachta indica* (Part 11)<sup>1</sup>: Characterisation and structure activity relationships of some novel rearranged azadirachtins. Tetrahedron. 47: 9231-9246.

- Ley, S. V., Somovilla, A. A., Broughton, H. B., Craig, D., Slawin, A. M. Z., Toogood, P. L. & Williams, D. J. (1989b). Chemistry of insect antifeedants from *Azadirachta indica* (Part 4): synthesis towards the limonoid azadirachtin; preperation of a functionalised decalin fragment. Tetrahedron. 45: 2143-2164.
- Ley, S. V. & Toogood, P. L. (1990). Insect antifeedants. Chem. Brit. Jan: 31-36.
- Li, H., Sheng, G., Teppen, B. J., Johnston, C. T. & Boyd, S. A. (2003). Sorption and desorption of pesticides by clay minerals and humic acid-clay complexes. Soil Sci. Soc. Am. J. 67: 122-131.
- Liang, G. M., Chen, W. & Liu, T. X. (2003). Effects of three neem-based insecticides on diamondback moth (lepidoptera: plutellidae). Crop Prot. 22: 333-340.
- Liu, B., McConnell, L. L. & Torrents, A. (2001). Hydrolysis of chlorpyrifos in natural waters of the Chesapeake Bay. Chemosphere. 44: 1315-1323.
- Longnecker, M. D., Rogan, W. J. & Lucier, G. (1997). The human effects of DDT (dichlorodiphenyltrichloroethane) & PCBS (polychlorinated biphenyls) on an overview of organochlorines in public health. Annu. Rev. Public Health. 18: 211-244.
- Lotti, M. (2002). Low-level exposure to organophosphorus esters and peripheral nerve function. Muscle Nerve. 25: 492-504.
- Lowery, D. T. & Isman, M. B. (1994a). Effects of neem and azadirachtin on aphids and their natural enemies, in Hedin, P. A. (ed.) Bioregulator Induced Effects on Crop Protection and Pest Resistance. ACS Symposium Series.
- Lowery, D. T. & Isman, M. B. (1994b). Insect growth regulating effects of neem extract and azadirachtin on aphids. Entomol. Exp. Appl. 72: 77-84.
- Luo, X., Ma, Y., Wu, S. & Wu, D. (1999). Two novel azadirachtin derivatives from *Azadirachta indica*. J. Nat. Prod. 60: 336-341.

- Ma, Q. L., Holland, P. T., James, T. K., McNaughton, D. E. & Rahman, A. (2000). Persistence and leaching of the herbicides acetochlor and terbutylazine in an allophanic soil: comparisons of field results with PRZM-3 predictions. Pest Manag. Sci. 56: 159-167.
- Maredia, K. M., Segura, O. L. & Mihm, J. A. (1992). Effects of neem, *Azadirachta indica*, on six species of maize insect pests. Tropical Pest Manag. 38: 190-195.
- Margni, M., Rossier, D., Crettaz, P. & Jolliet, O. (2002). Life cycle impact assessment of pesticides on human health and ecosystems. Agric. Eco. Environ. 93: 379-392.
- Martins, J. M. & Mermoud, A. (1998). Sorption and degradation of four nitroaromatic herbicides in mono and multi-solute saturated/unsaturated soil batch systems. J. Cont. Hydrol. 33: 187-210.
- Matthews, G. A. (2000). Pesticide Application Methods. Blackwell Science, UK.
- Matzinos, P., Tserki, V., Kontoyiannis, A. & Panayiotou, C. (2002). Processing and characterization of starch/polycaprolactone products. Pol. Deg. Stab. 77: 17-24.
- McCall, P. J., Laskowski, D. A., Swann, R. L. & Dishburger, H. J. (1980). Measurements of sorption coefficients of organic chemicals and their use in environmental fate analysis. Proc. Assoc. Offic. Anal. Chem. 94: 89-109.
- McLaren, J. S. (1986). Biologically active substances from higher plants: status and future potential. Pestic. Sci. 71: 559-578.
- McLaren, J. S. (2000). The importance of genomics to the future of crop production. Pest Manag. Sci. 56: 573-579.
- Metcalf, R. L. (1989). Insect resistance to insecticides. Pestic. Sci. 26: 333-358.



- Meyer-Windel, S. & Lennartz, B. (1995). Herbicide transport in differently structured soil horizons under constant and transient flow conditions, pp99-104, in Walker, A., Allen, R., Bailey, S. W., Blair, A. M., Brown, C. D., Günther, P., Leake, C. R. & Nicholls, P. H. (eds.) Pesticide Movement to Water. BCPC Monograph No. 62. British Crop Protection Council, Surrey, UK.
- Mogul, M. G., Akin, H., Hasirci, N., Trantolo, D. J., Gresser, J. D. & Wise, D. L. (1996). Controlled release of biologically active agents for purposes of agricultural crop management. Resources, Conservation and Recycling. 16: 289-320.
- Monmouth, (2003). General Mosquito Biology. Monmouth County Mosquito Extermination Commission. <http://www.visitmonmouth.com>
- Moody, K., Kust, C. A. & Buchholtz, K. P. (1970). Uptake of herbicides by soybean roots in culture solution. Weed Sci. 18: 642-647.
- Mordue (Luntz), A. J. (1997). Azadirachtin – A review of its mode of action in insects, pp1-4, in Kleeberg, H. (ed.) Practice Orientated Results on Use and Production of Neem-Ingredients and Pheromones. Neem Ingredients: Toxicological Results and Possible Medical Uses. Proc. 7<sup>th</sup> Workshop, Wetzlar, Germany, June 20-21, 1997.
- Mordue (Luntz), A. J. & Blackwell, A. (1993). Azadirachtin: an update. J. Ins. Physiol. 39: 903-924.
- Morgan, E. D. & Jarvis, A. P. (2001). Towards the total synthesis of azadirachtin, pp18-22, in Cole, M. & Strang, R. (eds.) The Science and Application of Neem 2001. Glasgow.
- Morrica, P., Giordano, A., Seccia, S., Ungaro, F. & Ventriglia, M. (2001). Degradation of imazosulfuron in soil. Pest Manag. Sci. 57: 360-365.

- Muda, R. & Cribb, B. W. (1999). Effect of uneven application of azadirachtin on reproductive and anti-feedant behaviour of *Rhyzopertha dominica* (Coleoptera: Bostichidae). Pestic. Sci. 55: 983-987.
- Mulla, M. S. & Su, T. (1999). Activity and biological effects of neem products against arthropods of medical and veterinary importance. J. Amer. Mosquito Cont. Assoc. 15: 133-152.
- Müller, M. (1997). Quantum chemical modelling of soil sorption coefficients: multiple linear regression models. Chemosphere. 35: 365-377.
- Naqvi, S. N. H. (1986). Biological evaluation of fresh neem extracts and some neem components, with reference to abnormalities and esterase activity in insects, pp315-330, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Narahashi, T. (2000). Neuroreceptors and ion channels as the basis for drug action: past, present, and future. J. Pharmacol. Exp. Therapeutics. 294: 1-26.
- National Research Council. (1992). Neem: A Tree for Solving Global Problems. National Academy Press, Washington, D.C.
- Neale, M. (2000). The regulation of natural products as crop-protection agents. Pest Manag. Sci. 56: 677-680.
- Nemeth-Konda, L., Füleky, G., Morovjan, G. & Csokan, P. (2002). Sorption behaviour of acetochlor, atrazine, carbendazim, diazinon, imidacloprid and isoproturon on Hungarian agricultural soil. Chemosphere. 48: 545-552.
- Nennemann, A., Mishaël, Y., Nir, S., Rubin, B., Polubesova, T., Bergaya, F., van Damme, H. & Lagaly, G. (2001). Clay-based formulations of metolachlor with reduced leaching. Appl. Clay Sci. 18: 265-275.
- Nicholls, P. H. (1988). Factors influencing entry of pesticides into soil water. Pestic. Sci. 22: 123-137.

- Nicholls, P., Campbell, A. T. & Williams, R. H. (2000). Factors influencing rates of degradation of an arylamide and a benzioc acid in subsoils. Pest Manag. Sci. 56: 607-614.
- Nisbet, A. J. (1992). The effects of azadirachtin on the feeding behaviour and virus transmission of the green peach aphid *Myzus persicae* (Sulzer). Ph.D. thesis, University of Glasgow. U. K.
- Nishioka, M. G., Burkholder, H. M., Brinkman, M. C. & Lewis, R. G. (1999). Distribution of 2,4-dichlorophenoxyacetic acid in floor dust throughout homes following homeowner and commercial lawn applications: quantitative effects of children, pets, and shoes. Environ. Sci. Technol. 33: 1359-1365.
- Norten, E. (2000). Neem: India's Miraculous Healing Plant. Healing Arts Press, Rochester, Vermont.
- Novak, S. M., Portal, J. M. & Schiavon, M. (2001). Effects of soil type upon metolachlor losses in subsurface drainage. Chemosphere. 42: 235-244.
- Oi, M. (1999). Time-dependent sorption of imidacloprid in two different soils. J. Agric. Food Chem. 47: 327-332.
- Oo, L. T. (1987). Neem tree research (terminal report). Burmese- German plant protection and rodent control project, Rangoon. Tana Press, Bangkok, Thailand, pp105.
- Parmar, B. S. (1986). An overview of neem research and use in India during 1983-1986, pp55-80, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Peng, J., Shen, X., El Sayed, K. A., Dunbar, D. C., Perry, T. L., Wilkins, S. P. & Hamann, M. T. (2003). Marine natural products as prototype agrochemical agents. J. Agric. Food Chem. 51: 2246-2252.

- Pepperman, A. B. & Kuan, J. W. (1992). HPLC and GC/MS of metribuzin and its degradation products from alginate-linseed oil controlled release formulations. J. Liquid Chrom. 15: 819-834.
- Pepperman, A. B. & Kuan, J. C. W. (1993). Slow release formulations of metribuzin based on alginate-kaolin-linseed oil. J. Controlled Release. 26: 21-30.
- Pepperman, A. B. & Kuan, J. C. W. (1995). Controlled release formulations of alachlor based on calcium alginate. J. Controlled Release. 34: 17-23.
- Pepperman, A. B., Kuan, J. C. W. & McCombs, C. (1991). Alginate controlled release formulations of metribuzin. J. Controlled Release. 17: 105-112.
- Pesticide Action Network UK. (2001). Guide to active ingredient hazards. <http://www.pan-uk.org>
- Peterson, C. J., Tsao, R. & Coats, J. R. (2000). Naturally occurring cyanohydrins, analogues and derivatives as potential insecticides. Pest Mang. Sci. 56: 615-617.
- Phillips, T. W. (1997). Semiochemicals of stored-product insects: research and applications. J. Stored. Prod. Res. 33: 17-30.
- Pimentel, D., Acquay, H., Biltonen, M., Rice, P., Silva, M., Nelson, J., Lipner, V., Giordano, S., Horowitz, A. & D'Amore, M. (1992). Environmental and human costs of pesticide use. Bioscience. 42: 750-760.
- Pimentel, D., Krummel, J., Gallahan, D., Hough, J., Merrill, A., Schreiner, I., Vittum, P., Koziol, F., Back, E., Yen, D. & Fiance, S. (1981). A cost-benefit analysis of pesticide use in the U. S. food production, pp27-54, in Pimentel, D. (ed.) Handbook of Pest Management in Agriculture (Vol 2). CRC Press, Boca Raton, Florida.
- Plapp (Jr), F. W. (1981). The nature, modes of action, and toxicity of insecticides, pp3-16, in Pimentel, D. (ed.) Handbook of Pest Management in Agriculture (Vol 3). CRC Press, Boca Raton, Florida.



- Powell, W. & Pickett, J. A. (2003). Manipulation of parasitoids for aphid pest management: progress and prospects. Pest Manag. Sci. 59: 149-155.
- Prabhaker, N., Coudriet, D. L., Kishaba, A. N. & Meyerdirk, D. E. (1986). Laboratory evaluation of neem-seed extract against larvae of the Cabbage Looper and Beer Armyworm (Lepidoptera: Noctuidae). J. Econ. Entomol. 79: 39-41.
- Puri, H. S. (1999). Neem: The Divine Tree *Azadirachta indica*. Harwood Academic Publishers, Netherlands.
- Qi, B., Gordon, G. & Gimme, W. (2001). Effects of neem-fed prey on the predacious insects *Harmonia conformis* (Boisduval) (Coleoptera: Coccinellidae) and *Mallada signatus* (Schneider) (Neuroptera: Chrysopidae). Biol. Cont. 22: 185-190.
- Qian, H., Bei, J. & Wang, S. (2000). Synthesis, characterization and degradation of ABA block copolymer of L-lactide and  $\epsilon$ -caprolactone. Pol. Deg. Stab. 68: 423-429.
- Quaglia, F., Barbato, F., de Rosa, G., Granata, E., Miro, A. & la Rotonda, M. I. (2001). Reduction of the environmental impact of pesticides: waxy microspheres encapsulating the insecticide carbaryl. J. Agric. Food Chem. 49: 4808-4812.
- Radwanski, S. A. (1977). Neem tree. I. Commercial potential characteristics and distribution. World Crop Livestock. 29: 22-66.
- Radwanski, S. A. & Wickens, G. E. (1981). Vegetative fallows and potential value of the neem tree (*Azadirachta indica*) in the tropics. Econ. Bot. 35: 398-414.
- Raizada, R. B., Srivastava, M. K., Kaushal, R. A. & Singh, R. P. (2001). Azadirachtin, a neem biopesticide: subchronic toxicity assessment in rats. Food Chem. Tox. 39: 477-483.
- Raveton, M., Ravanel, P., Serre, A. M., Nurit, F. & Tissut, M. (1997). Kinetics of uptake and metabolism of atrazine in model plant systems. Pestic. Sci. 49: 157-163.

- Rawat, G. S. (1995). Neem (*Azadirachta indica*) – nature's drugstore. Indian Forester. 121: 977-980.
- Redfern, R. E., Hayes, D. K., Warthen, J. D. Jr., de Milo, A. B. & McGovern, T. P. (1984). Responses of nymphs of the large milkweed bug and pupae of the yellow mealworm to three compounds affecting insect growth. Ann. Rev. Chronopharmac. 1: 239-242.
- Rembold, H. (1989). Azadirachtin: Their structure and mode of action, pp150-163, in Arnason, J. T., Philogene, B. J. R. & Morand, P. (eds.) Insecticides of Plant Origin. ACS symp. Ser. 387. American Chemical Society, Washington, D. C.
- Rembold, H., Forster, H. & Czoppelt, C. H. (1986). Structure and biological activity of azadirachtins A and B, pp149-160, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Rembold, H., Forster, H., Czoppelt, C. H., Rao, P. J. & Sieber, K. P. (1983). The azadirachtins, a group of insect growth regulators from the neem tree, pp153-162, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 2<sup>nd</sup> Int. Neem Conf. Rauschholzhausen, Germany.
- Rembold, H., Uhl, M. & Muller, T. H. (1986) Effect of azadirachtin A on hormone titers during the gonadotrophic cycle of *Locusta migratoria*, pp289-298, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Renwick, A. G. (2002). Pesticide residue analysis and its relationship to hazard characterisation (ADI/ARfD) and intake estimations (NEDI/NESTI). Pest Manag. Sci. 58: 1073-1082.
- Richardson, M. (1975). Translocation in Plants (2<sup>nd</sup> Ed.). Edward Arnold, London.
- Ritter, L. (1997). Report of a panel on the relationship between public exposure to pesticides and cancer. Cancer. 10: 2019-2033.

- Robinson, R. A. & Sutherland, W. J. (2002). Post – war changes in arable farming and biodiversity in Great Britain. J. Appl. Ecol. 39: 157-176.
- Ross, J. H., Dong, M. H. & Krieger, R. I. (2000). Conservatism in pesticide exposure assessment. Reg. Tox. Pharmacol. 31: 53-58.
- Rotteveel, T. J. W., de Goeij, J. W. F. M. & van Gemerden, A. F. (1997). Towards the construction of a resistance risk evaluation scheme. Pestic. Sci. 51: 407-411.
- Roush, R. T. & Daly, J. C. (1990). The role of population genetics in resistance research and management, pp97-152, in Roush, R. T. & Tabashnik, B. E. (eds.) Pestic. Resist. Arthropods. Chapman & Hall, New York.
- Roy, C., Gaillardon, P. & Montfort, F. (2000). The effect of soil moisture content on the sorption of five sterol biosynthesis inhibiting fungicides as a function of their physicochemical properties. Pest Manag. Sci. 56: 795-803.
- Salehzadeh, A., Jabbar, A., Jennens, L., Ley, S. V., Annadurai, R. S., Adams, R. & Strang, R. H. C. (2001). The effects of phytochemical pesticides on the growth of cultured invertebrate and vertebrate cells. Pest Manag. Sci. 58: 268-276.
- Sales, K. M., Kingston, S. T., Atterwill, C. K. & Purcell, W. M. (2000). Avian whole-brain spheroid cultures: applications in pesticide toxicity. Pest Manag. Sci. 56: 825-827.
- Sánchez-Brunete, C., Rodriguez, A. & Tadeo, J. L. (2003). Multiresidue analysis of carbamate pesticides in soil by sonication-assisted extraction in small columns and liquid chromatography. J. Chrom. A. In press.
- Sanchez-Hernandez, J. C. & Walker, C. H. (2000). *In vitro* and *in vivo* cholinesterase inhibition in lacertides by phosphonate- and phosphorothioate-type organophosphates. Pestic. Biochem. Physiol. 67: 1-12.

SANCO (2001). Report from the commission to the European parliament and the council. Evaluation of the active substances of plant protection products (submitted in accordance with article 8(2) of Council Directive 91/414/EEC on the placing of plant protection products on the market. Commission of the European Communities. 12 July 2001, Review 3: 822/2001.

SANCO (2003). Guidance document on the assessment of the relevance of metabolites in groundwater of substances regulated under Council Directive 91/414/EEC. European Commission. 25 February 2003, Review 10: 221/2000.

Saxena, R. C. (1986). Neem seed derivatives for management of rice insect pests – A review of recent studies, pp81-93, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.

Saxena, R. C. (2002). Hemiptera / Homoptera: Leaf- and planthoppers, aphids, psyllids, whiteflies and scale insects, pp342-365, in Schmutterer, H. (ed.) The Neem Tree, *Azadirachta indica* A. Juss. and Other Meliaceae Plants. Sources of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes (2<sup>nd</sup> Ed.). Neem Foundation, Vithalnagar, Juhu Scheme, Mumbai, 2002.

Saxena, R. C., Jilani, G. & Kareem, A. (1988). Effects of neem on stored grain insects, pp97-111, in Jacobson, M. (ed.) Focus on Phytochemical Pesticides (Vol. 1) The Neem Tree. CRC Press, Boca Raton, Florida.

Saxena, R. C., Liquido, N. J. & Justo, H. D. (1981). Neem seed oil, a potential antifeedant for the control of the rice brown planthopper, *Nilaparvata lugens*, pp171-180, in Schmutterer, H., Ascher, K. R. S. & Rembold, H. (eds.) Proc. 1<sup>st</sup> Int. Neem Conf. Rottach-Egern.

Schaaf, O., Jarvis, A. P., Van der Esch, S. A., Giagnacovo, G. & Oldham, G. (2000). Rapid and sensitive analysis of azadirachtin and related triterpenoids from neem (*Azadirachta indica*) by high-performance liquid chromatography – atmospheric pressure chemical ionization mass spectrometry. J. Chrom. 886: 89-97.



Schjønning, P., Elmholt, S., Munkholm, L. J. & Debosz, K. (2002). Soil quality aspects of humid sandy loams as influenced by organic and conventional long-term management. Agric. Eco. Environ. 88: 195-214.

Schlüter, U. (1986). Effects of azadirachtin on developing tissues of various insect larvae, pp331-350, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.

Schmutterer, H. (1986). Fecundity-reducing and sterilizing effects of neem seed kernel extracts in the Colorado potato beetle, *Leptinotarsa decemlineata*, pp351-360, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.

Schmutterer, H. (1990). Properties and potential of natural pesticides from the neem tree, *Azadirachta indica*. Ann. Rev. Ent. 35: 271-297.

Schmutterer, H. (1995). Thysanoptera: thrips, pp51-254, in Schmutterer, H. (ed.) The Neem Tree, *Azadirachta indica* A. Juss. and Other Meliaceae Plants. Sources of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes. VCH Verlagsgesellschaft mbH, Germany.

Schmutterer, H. (2002). The tree and its characteristics, pp1-34, in Schmutterer, H. (ed.) The Neem Tree, *Azadirachta indica* A. Juss. and Other Meliaceae Plants. Sources of Unique Natural Products for Integrated Pest Management, Medicine, Industry and Other Purposes (2<sup>nd</sup> Ed.). Neem Foundation, Vithalnagar, Juhu Scheme, Mumbai, 2002.

Schoonhoven, L. M. (1982). Biological aspects of antifeedants. Entomol. Exp. Appl. 31: 57-69.

Schroeder, D. R. & Nakanishi, K. (1987). A simplified isolation procedure for azadirachtin. J. Nat. Prod. 50: 241-244.

Schwartz P. H. & Klassen, W. (1981). Estimate of losses caused by insect and mites to agricultural crops, pp15-77, in Pimentel, D. (ed.) Handbook of Pest Management in Agriculture (Vol 1). CRC Press, Boca Raton, Florida.

Scott, J. G., Alefantis, T. G., Kaufman, P. E. & Rutz, D. A. (2000). Insecticide resistance in house flies from caged-layer poultry facilities. Pest Manag. Sci. 56: 147-153.

SETAC (1995). Procedures for Assessing the Environmental Fate and Ecotoxicity of Pesticides. Lynch, M. R. (ed.) Society of Environmental Toxicology and Chemistry, Belgium.

Settimi, L., Masina, A., Andrion, A. & Axelson, O., (2003). Prostate cancer and exposure to pesticides in agricultural settings. Int. J. Cancer. 104: 458-461.

Sharom, M. S., McEwen, F. L. & Harris, C. R. (1981). Movement of insecticides in the environment and biodegradability, pp143-166, in Pimentel, D. (ed.) Handbook of Pest Management in Agriculture (Vol 3). CRC Press, Boca Raton, Florida.

Shimizu, T. (1988). Suppressive effects of azadirachtin on spermatogenesis of the diapausing cabbage armyworm, *Mamestra brassicae*, in vitro. Entomol. Exp. Appl. 46: 197-199.

Sicbaldi, F., Sacchi, G. A., Trevisan, M. & Re, A. A. M. D. (1997). Root uptake and xylem translocation of pesticides from different chemical classes. Pestic. Sci. 50: 111-119.

Siddiqui, S. (1942). A note on the isolation of three new bitter principles from the nim oil. Curr. Sci. 11: 278-279.

Sidhu, O. P., Kumar, V. & Behl, H. M. (2003). Variability in neem (*Azadirachta indica*) with respect to azadirachtin content. J. Agric. Food Chem. 51: 910-915.

- Simmonds, M. S. J. & Blaney, W. M. (1983). Some neurophysiological effects of azadirachtin on lepidopterous larvae, pp163-180, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 2<sup>nd</sup> Int. Neem Conf. Rauschholzhausen, Germany.
- Simmonds, M. S. J., Blaney W. M., Ley, S. V., Anderson, J. C. & Toogood P. L. (1990). Azadirachtin: structural requirements for reducing growth and increasing mortality in lepidopterous larvae. Entomol. Exp. Appl. 55: 169-181.
- Singh, R. P. (1986). Comparison of antifeedant efficacy and extract yields from different parts and ecotypes of neem (*Azadirachta indica* A. Juss) trees, pp185-194, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Singh, U. S. (1989). Studies on the systemicity of [<sup>14</sup>C] metalaxyl in cowpea (*Vigna unguiculata* (L.) Walp.). Pestic. Sci. 25: 145-154.
- Sinniah, D., Baskaran, G., Looi, L. M. & Leong, K. L. (1982). Reye-like syndrome due to Margosa oil poisoning: report of a case with postmortem findings. Am. J. Gastroenterol. 77: 158-161.
- Sivalingam, G. & Madras, G. (2003). Thermal degradation of poly(ε-caprolactone). Pol. Deg. Stab. 80: 11-16.
- Solvay Interlox Ltd (1998). Biodegradable CAPA® thermoplastics. Solvay Internal Report. March: 1-7.
- Spark, K. M. & Swift, R. S. (2002). Effect of soil composition and dissolved organic matter on pesticide sorption. Sci. Total Environ. 298: 147-161.
- Stapley, J. H. & Gayner, F. C. H. (1969). World crop protection: pests and diseases (Vol 1). Illiffe books, London.
- Stark, J. D. & Walter, J. F. (1995a). Persistence of azadirachtin A and B in soil: effects of temperature and microbial activity. J. Environ. Sci. Health. B30: 685-698.

- Stark, J. D. & Walter, J. F. (1995b). Neem oil and neem oil components affect the efficacy of commercial neem insecticides. J. Agric. Food Chem. 43: 507-512.
- Stevens, P. J. G. & Baker, E. A. (1987). Factors affecting the foliar absorption and redistribution of pesticides. 1. Properties of leaf surfaces and their interactions with spray droplets. Pestic. Sci. 19: 265-281.
- Stickel, W. H., Stickel, L. F., Dryland, R. A. & Hughes, D. L. (1984). DDT in birds: lethal residues and loss rates. Archives Environ. Contam. Tox. 13: 1-6.
- Stoate, C., Boatman, N. D., Borralho, R. J. Carvalho, C. R., de Snoo, G. R. & Eden, P. (2001). Ecological impacts of arable intensification in Europe. J. Environ. Manag. 63: 337-365.
- Storm, J. E., Rozman, K. K. & Doull, J. (2000). Occupational exposure limits for 30 organophosphate pesticides based on inhibition of red blood cell acetylcholinesterase. Toxicology. 150: 1-29.
- Sugavanam, B. & Copping, L. G. (1998). Development of crop protection agents – invention to sales, pp3-29, in van Valkenburg, W; Sugavanam, B. & Khetan, K. S. (eds.) Pesticide Formulation: Recent Developments and their Applications in Developing Countries. New Delhi, India.
- Sundaram, K. M. S. (1996). Root uptake, translocation, accumulation and dissipation of the botanical insecticide, azadirachtin in young spruce trees. J. Environ. Sci. Health. B31: 1289-1306.
- Sundaram K. M. S. & Curry, J. (1996). Effect of some UV light absorbers on the photostabilization of azadirachtin, a neem-based biopesticide. Chemosphere. 32: 649-659.
- Taiz, L. & Zeiger, E. (1998). Plant Physiology (2<sup>nd</sup> Ed.). Sinauer Associates, Inc., Publishers, Sunderland, Massachusetts.



Tatter, T. A. (2003). Fighting infestation: microinjection can help save eucalyptus trees. [http:// www.looksmart.com](http://www.looksmart.com)

Taylor, D. A. H. (1987). Azadirachtin: a study in the methodology of structure determination. Tetrahedron. 43: 2779-2787.

Tedeschi, R., Alma, A. & Tavella, L. (2001). Side-effects of three neem (*Azadirachta indica* A. Juss) products on the predator *Macrolphus caliginosus* Wagner (Het., Miridae). J. Appl. Ent. 125: 397-402.

Thejavathi, R., Yakkundi, S. R. & Ravindranath, B. (1995). Determination of azadirachtin by reversed-phase high performance liquid chromatography using anisole as internal standard. J. Chrom. A. 705: 374-379.

Thöming, G., Borgemeister, C. & Poehling, H. M. (2003). Systemic effects of neem on western flower thrips, pp20-25, in Cole, M. & Strang, R. (eds.) The Science & Application of Neem 2003.

Thompson, G. D., Dutton, R. & Sparks, T. C. (2000). Spinosad – a case study: an example from natural products discovery programme. Pest Manag. Sci. 56: 696-702.

Timmins, W. A. & Reynolds, S. E. (1992). Azadirachtin inhibits secretion of trypsin in midgut of *Manduca sexta* caterpillars: reduced growth due to impaired protein digestion. Entomol. Exp. Appl. 63: 47-54.

Tomlin, C (ed.) (1994). The pesticide manual: incorporating the agrochemical handbook (10<sup>th</sup> Ed.) The British Crop Protection Council, Surrey, and The Royal Society of Chemistry, Cambridge, UK.

Tomlin, C (ed.) (1997). The pesticide manual: incorporating the agrochemical handbook (11<sup>th</sup> Ed.) The British Crop Protection Council, Surrey, UK.

- Toth, S. J. & Sparks, T. C. (1990). Effect of temperature on toxicity and knockdown activity of *cis*-permethrin, esfenvalerate and lambda-cyhalothrin in the cabbage looper (Lepidoptera: Noctuidae). J. Econ. Entomol. 83: 342-346.
- Trapp, S. (2000). Modelling uptake into roots and subsequent translocation of neutral and ionisable organic compounds. Pest. Manag. Sci. 56: 767-778.
- Trimnell, D., Shasha, B. S., Wing, R. E. & Otey, F. H. (1982). Pesticide encapsulation using a starch-borate complex as wall material. J. Appl. Pol. Sci. 27: 3919-3928.
- Turner, C. J., Tempesta, M. S., Taylor, R. B., Zagorski, M. G., Termini, J. S., Schroeder, D. R. & Nakanishi, K. (1987). An nmr spectroscopic study of azadirachtin and its trimethyl ether. Tetrahedron. 43: 2789-2803.
- Tyree, M. T., Peterson, C. A. & Edgington, L. V. (1979). A simple model regarding ambimobility with special reference to the nematocide, oxamyl. Plant Physiol. 63: 367-374.
- Unsworth, Wauchope, Klein, Dorn, Zeeh, Yeh, Akerblom, Racke & Rubin. (1999). Pesticide report 41. Significance of the long range transport of pesticides in the atmosphere (Technical Report). Pure App. Chemis. 71: 1359-1383.
- US EPA, (2002). Pesticides and Mosquito Control. <http://www.epa.gov>
- Vais, H., Williamson, M. S., Devonshire, A. L. & Usherwood, P. N. R. (2001). The molecular interactions of pyrethroid insecticides with insect and mammalian sodium channels. Pest Manag. Sci. 57: 877-888.
- Vale, C., Fonfría, E., Bujons, J., Messeguer, A., Rodríguez-Farré, E. & Suñol, C. (2003). The organochlorine pesticides  $\gamma$ -hexachlorocyclohexane (lindane),  $\alpha$ -endosulfan and dieldrin differentially interact with GABA<sub>A</sub> and glycine-gated chloride channels in primary cultures of cerebellar granule cells. Neuroscience. 117: 397-403.

- van Beek, T. A. & de Groot, A. (1986). Terpenoid antifeedants, part 1. An overview of terpenoid antifeedants of natural origin. Recl. Trav. Chim. Pays-Bas. 105: 513-527.
- van der Nat, J. M., van der Sluis, W. G., de Silva, K. T. D. & Labadie, R. P. (1991). Enthopharmacognostical survey of *Azadirachta indica* A. Juss (Meliaceae). J. Ethnopharmacol. 35: 1-24.
- van der Werf, H. M. G. (1996). Assessing the impact of pesticides on the environment. Agric. Eco. Environ. 60: 81-96.
- Vaughn, M. W., Harrington, G. N. & Bush, D. R. (2002). Sucrose-mediated transcriptional regulation of sucrose symporter activity in the phloem. Proc. Natl. Acad. Sci. 99: 10876-10880.
- Vieira, E. D. R., Torres, J. P. M. & Malm, O. (2001). DDT environmental persistence from its use in a vector control program: a case study. Environ. Res. Section A. 86: 174-182.
- Villatte, F. & Bachmann, T. T. (2002). How many genes encode cholinesterase in arthropods? Pestic. Biochem. Physiol. 73: 122-129.
- Völlinger, M. (1986). The possible development of resistance against neem seed kernel extract and deltamethrin in *Plutella xylostella*, pp543-554, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Walker, A. & Allen, R. (1984). Influence of soil and environmental factors on persistence, pp89-100, in Hance, R. J. (ed.) Soils and Crop Protection Chemicals. Monograph No. 27. Proceedings of a symposium held at Wye College, Kent. 11<sup>th</sup> and 12<sup>th</sup> July, 1984. BCPC Publications, Croydon.
- Waller, J. M. (1985). Plant disease control, pp427-457, in Haskell, P. T. (ed.) Pesticide Application: Principles and Practice. Clarendon Press, Oxford.

- Walter, A., Welch, S. J. & Turner, I. J. (1995). Studies of time-dependent sorption processes in soils, pp13-18, in Walker, A., Allen, R., Bailey, S. W., Blair, A. M., Brown, C. D., Günther, P., Leake, C. R. & Nicholls, P. H. (eds.) Pesticide Movement to Water. BCPC Monograph No. 62. British Crop Protection Council, Surrey, UK.
- Wang, L., Ma, W., Gross, R. A. & McCarthy, S. P. (1998). Reactive compatibilization of biodegradable blends of poly(lactic acid) and poly( $\epsilon$ -caprolactone). Pol. Deg. Stab. 59: 161-168.
- Wang, Q., Yang, W. & Liu, W. (1999). Adsorption of acetanilide herbicides on soils and its correlation with soil properties. Pestic. Sci. 55: 1103-1108.
- Warrior, P. (2000). Living systems as natural crop-protection agents. Pest Mang. Sci. 56: 681-687.
- Warthen, J. D., Stokes, J. B., Jacobson, M. & Kozempel, M. F. (1984). Estimation of azadirachtin content in neem extracts and formulations. J. Liquid Chrom. 7: 591-598.
- Wauchope, R. D., Yeh, S., Linders, J. B. H. J., Kloskowski, R., Tanaka, K., Rubin, B., Katayama, A., Kördel, W., Gerstl, Z., Lane, M. & Unsworth, J. B. (2002). Pesticide soil sorption parameters: theory, measurement, uses, limitations and reliability. Pest Manag. Sci. 58: 419-445.
- Webster, L. R., McKenzie, G. H. & Moriarty, H. T. (2002). Organophosphate-based pesticides and genetic damage implicated in bladder cancer. Cancer Gen. Cytogen. 133: 112-117.
- White, R. E. (1979). Introduction to the Principles and Practice of Soil Science. Blackwell Scientific Publications, Oxford, London, Edinburgh, Melbourne.
- Wieneke, J. & Steffens, W. (1976). Translocation and metabolism of azinphos-methyl in bean plants after root and leaf absorption. J. Agric. Food Chem. 24: 416-418.



- Wilkinson, C. F. (1984). Metabolism and selective toxicity in an environmental context, pp133-147, in Caldwell, J. & Paulson, G. D. (eds.) Foreign Compound Metabolism. Taylor & Francis, London & Philadelphia.
- Wilps, H. (1986). Growth and adult moulting of larvae and pupae of the blowfly *Phormia terrae-novae* in relationship to azadirachtin concentrations, pp299-314, in Schmutterer, H. & Ascher, K. R. S. (eds.) Proc. 3<sup>rd</sup> Int. Neem Conf. Nairobi.
- Wilson, C. & Tisdell, C. (2001). Why farmers continue to use pesticides despite environmental, health and sustainability costs. Ecological Econ. 39: 449-462.
- Worrall, F., Fernandez-Perez, M., Johnson, A. C., Flores-Cesperedes, F. & Gonzales-Pradas, E. (2001). Limitations on the role of incorporated organic matter in reducing pesticide leaching. J. Cont. Hydrol. 49: 241-262.
- Xu, F., Liang, X. M., Su, F., Zhang, Q., Lin, B. C., Wu, W. Z., Yediler, A. & Kettrup, A. (1999). A column method for determination of soil organic partition coefficients of eight pesticides. Chemosphere. 39: 787-794.
- Yamasaki, R. B., Klocke, J. A., Lee, S. M., Stone, G. A. & Darlington, M. V. (1986). Isolation and purification of azadirachtin from neem (*Azadirachta indica*) seeds using flash chromatography and high performance liquid chromatography. J. Chrom. 356: 220-226.
- Yavuz, H., Babaç, C., Tuzlakoglu, K. & Pişkin, E. (2002). Preparation and degradation of L-lactide and  $\epsilon$ -caprolactone homo and copolymer films. Pol. Deg. Stab. 75: 431-437.
- Yerushalmi, N. & Cohen, E. (2002). Acetylcholinesterase of the California red scale *Aonidiella aurantii* Mask.: Catalysis, inhibition, and reactivation. Pestic. Biochem. Physiol. 72: 133-141.

Zanno, P. R., Miura, I., Nakanishi, K. & Elder, D. L. (1975). Structure of the phagorepellent azadirachtin. Application of PRFT/CWD carbon-13 nuclear magnetic resonance. J. Am. Chem. Soc. 97: 1975-1977.

Zhu, K. Y. & Gao, J. R. (1999). Increased activity associated with reduced sensitivity of acetylcholinesterase in organophosphate – resistant greenbug, *Schizaphis graminum* (Homoptera: Aphididae). Pestic. Sci. 55: 11-17.